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University College London
Windeyer Institute of Medical Sciences

Particle-mediated DNA delivery:
Evaluation of parameters affecting efficacy
in vivo and in vitro

A thesis submitted by

Patrick Gilboy

for the degree of
Doctor of Philosophy
in the University of London
2005

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Abstract

Particle-mediated DNA delivery (PMDD) is a technique that uses compressed helium gas to accelerate microscopic gold beads coated with DNA into the skin. As a vaccination tool, it provides an attractive approach for generating antigen specific immunotherapy. As the number of applications for this technology in pre-clinical disease models grows, the need for *in vitro* surrogate models, which in part, can replace *in vivo* testing, is becoming increasingly important. Additionally, before it can be utilised successfully in man, a number of critical parameters which contribute to overall efficacy *in vivo* need to be more fully evaluated. To these ends, an *in vitro* model which featured PMDD as the means of plasmid delivery was developed. The relationship between the efficiency of PMDD to stimulate antigen specific CD8+ T cell responses *in vitro* and *in vivo* under conditions of single and dual plasmid immunisation was explored. Important aspects of the technology such as the association between antigen dose and resulting response were investigated. It was found that the *in vitro* model correlated well with the early stage primary response *in vivo* but this correlation deteriorated post boost. In addition, the relative contribution of antigenic competition and co-operation in shaping the resulting response was assessed after co-immunising with two independent antigens. Under the experimental conditions investigated, the dominant type of interaction was found to be competitive. Results suggested that competition occurred at the level of the T cell. Competition was most pronounced when both antigens were delivered in a linked manner and when both antigens were presented on the same APC. These results highlight the requirement for optimising key technical parameters in PMDD. Additionally, it demonstrates that interclonal T cell competition is a functionally relevant phenomenon and that it can occur when standard DNA vaccination strategies are employed.

To family and friends

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Abbreviations

AICD	activation induced cell death
APC	antigen presenting cells
BMDC	bone marrow derived dendritic cells
BSA	bovine serum albumin
CCL	C-C chemokine
CCR	C-C chemokine receptor
CMV	cytomegalovirus
CPS	counts per second
CTL	cytotoxic T cell
CXCL	C-X-C chemokine
CXCR	C-X-C chemokine receptor
DC	dendritic cell
DLR	DNA loading rate
DMEM	dulbeccos modified eagles medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide phosphate
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
FCS	foetal calf serum
GM-CSF	granulocyte/macrophage-colony stimulating factor
GSK	GlaxoSmithKline
GST	glutathione-S-transferase
HIV	human immunodeficiency virus
HPV	human papilloma virus
HRP	horse radish peroxidase
ICAM	intracellular adhesion molecule
IFN	interferon
IL	interleukin

IMDM	iscoves modified eagles medium
LC	Langherhans cell
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
NP	nucleoprotein
NT	northern territories
OVA	ovalbumin
PB	post boost
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMDD	particle-mediated DNA delivery
PP	post primary
PWO	<i>Pyrococcus woesei</i>
RLU	relative light units
RNG	HIV Reverse Transcriptase, Nef and Gag protein fusion
RNA	ribonucleic acid
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
TcR	T cell receptor
Th	T helper cell
TNF	tumour necrosis factor
TLR	Toll-like receptor
UCL	University College London

Chapter 1

General introduction

1.1. Introduction

The physiological function of the immune system is protection against infectious agents. In order to achieve this, the immune system has evolved sophisticated methods of recognising and eliminating these agents. The product of the immune system is termed the immune response and is broadly divided into two categories; the innate immune response and the adaptive or acquired immune response. They consist of uniquely different components and as separate mechanisms of defence, are extremely effective. The type and severity of invading pathogen dictates the most suitable type of response launched. More often than not, an effective immune response incorporates elements from both systems which interact in a co-ordinated and synchronised manner to remove the pathogen and restore health.

Vaccination provides a powerful tool to amplify and integrate aspects of both the innate and adaptive immune response in order to eliminate pathogens. In this regard, the emerging field of DNA vaccination employs a broad and varied number of strategies in order to elicit appropriate immune responses in prophylactic and therapeutic settings. Here, the contribution of one form of DNA vaccination; particle-mediated DNA delivery (PMDD) is discussed.

1.2. Innate immune response

Innate immunity is the phylogenetically oldest mechanism of defence against foreign pathogens. Through the last number of decades, it was widely believed to be relatively non-specific in its recognition and effector functions. However, recent evidence suggests that innate immunity is much more specific and well defined than first imagined (Janeway and Medzhitov, 2002). It is most important in the early stages of infection and is primarily mediated by sub-populations of leukocytes, particularly monocytes, macrophages and natural killer (NK) cells.

1.2.1. Innate recognition

Mononuclear and polymorphonuclear phagocytes possess a certain number of receptors which are dedicated to recognising conserved microbial molecules common to many microbial species (Kimbrell and Beutler, 2001). These molecules are usually indispensable components of the microbe which are not prone to alteration or mutation and thus constitute preserved targets for the innate response. The receptors used to recognise these targets are referred to as pattern recognition receptors (PRRs) and the structural features of the microbe which are recognised are the pathogen associated microbial patterns (PAMPs) (Werling and Jungi, 2003). One of the most well-known groups of proteins which act as PRRs are the Toll-like receptors (TLRs). These are type I integral membrane glycoproteins with ectodomains consisting of leucine rich repeats which form the PAMPs and a cytoplasmic tail sharing homology with the signalling domain of the IL-1 family (O'Neill, 2002). To date, the TLR family consists of 13 members, 10 of which (TLR1-TLR10) have been identified in humans (Chuang and Ulevitch, 2001). Of these, TLR1-TLR6 are cell surface expressed while TLR7-TLR9 are found intra-cellularly. They bind to a wide range of ligands such as peptidoglycan (TLR2), unmethylated DNA (TLR9), dsRNA (TLR3), ssRNA (TLR7), bacterial flagellin (TLR5) lipopolysaccharide (TLR4) and lipoteichoic acid (TLR2) (Netea *et al.*, 2004). While some of these receptors operate on their own, others combine with each other to form heterodimeric complexes. Examples of such complexes are those formed by TLR1/2 (Wetzler, 2003), TLR2/6 (Hajjar *et al.*, 2001) and TLR4/5 (Mizel *et al.*, 2003) which serve to extend the repertoire of specificities of molecules recognised.

Once activated, the predominant signalling pathway used by TLRs involves recruitment of cytoplasmic adapter proteins like myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs) and TNF-receptor-associated factor 6 (TRAF6) (Takeda and Akira, 2004). The activated signalling cascade causes the phosphorylation and degradation of $\text{I}\kappa\text{B}$ resulting in the nuclear translocation and activation of NF- κB . The genes that are expressed in response to TLR activation are cell line dependent but include anti-viral cytokines such as type I interferons, the inflammatory

cytokines IL-1, IL-12 and TNF- α as well as adhesion molecules (E-selectin) and proteins involved in microbial killing (reactive oxygen intermediates such as nitric oxide) (Ozato *et al.*, 2002).

Another family of proteins which recognise PAMPs are the nucleotide binding oligomerization domain (NOD) proteins. These are cytosolically localised proteins which recognise peptidoglycan structures that are not ligands for TLRs (Girardin *et al.*, 2003). Signalling events for these receptors are mediated by members of the caspases family and also result in activation of NF- κ B (Martinon and Tschopp, 2004). Other PRRs include mannose receptors which specifically recognise glycoproteins with terminal mannose or fucose residues and scavenger receptors which bind to oxidised or acetylated low density lipoproteins (Linehan *et al.*, 1999). Different types of G protein coupled receptors (also called seven transmembrane α -helical receptors) are expressed on leukocytes and bind to ligands containing *N*-formylmethionyl residues. Some G protein coupled receptors also bind to proteins of the complement cascade such as C5a (Vanek *et al.*, 1994), the neutrophil attractant IL-8 (Holmes *et al.*, 1991) and a range of inflammatory mediators including prostaglandin E (Fujino and Regan, 2003), leukotriene B₄ (Schepers and McLeish, 1993) and platelet activating factor (Lukashova *et al.*, 2001).

1.2.2. Innate effector function

Innate effector function is mediated by a number of components which may be broadly divided into two different categories. First is the cellular arm which includes the mononuclear/polymorphonuclear and NK cells. Once activated, neutrophils and macrophages kill the ingested microbes by the co-operative activity of synthesised reactive oxygen and nitrogen intermediates (Alvaraz-Dominguez *et al.*, 2000; Akaki *et al.*, 1997). These cell types also produce several proteolytic enzymes such as elastase which function to kill ingested microbes (Ribeiro-Gomez *et al.*, 2004). Macrophages fulfil the additional duties of recruiting other myeloid cells to the site of infection by release of TNF- α and IL-1 (Gao and Tsan, 2004) and activating NK and T cells by secretion of IL-12 (Xing *et al.*, 2000). NK cell activation is regulated by the balance of expression of stimulatory and

inhibitory receptors on its surface. Following activation, these cells destroy infected or malignant cells by interacting with the adaptive immune response in a process known as antibody dependant cellular cytotoxicity (ADCC) or by the sequential release of perforin and granzymes which create pores in the membrane of the target cell and induce apoptosis, respectively (Jie and Sarvetnick, 2004).

Second are the soluble mediators of the innate response which include plasma proteins and the complement system. These include the mannose binding proteins which opsonise carbohydrates with terminal mannose or fucose residues and activate the complement cascade via the lectin pathway (Super and Ezekowitz, 1992). The C reactive proteins bind to phospholipids of pneumococcal bacteria and activate the classical complement pathway via C1q ligation or may bind directly to Fc- γ receptors (Mortenson, 2001). Another important component of innate immunity which serves to remove foreign pathogens is a group of plasma (and cell surface) proteins collectively known as the complement system. There are three major pathways of complement activation all of which lead to the congregation of C6, C7, C8 and C9 proteins at the surface of the invading micro-organism. These proteins form a membrane attack complex (MAC) and result in lysis of the target micro-organism (Fujita *et al.*, 2004).

1.3. The adaptive immune response

While innate immunity is primarily characterised by lack of immunologic memory and to a lesser extent lack of specificity, these attributes are the signature of the adaptive response. It is mediated by lymphocytes (i.e. B and T cells), initiated by interaction with antigen presented to these cells by professional APCs such as DCs and improves with each and every antigenic encounter.

Specifically, this review focuses on the role of DCs in the adaptive immune response (Section 1.3.1), the activation of CD8⁺ and CD4⁺ T cells in the secondary lymphoid organs and the effector functions of these cells once activated (Sections 1.3.2 and 1.3.3, respectively). Additionally, the dichotomous relationship between CD8⁺ and CD4⁺ T cells

during the primary and memory phases of the immune response is discussed in Section 1.3.5 with particular emphasis on PMDD.

1.3.1. Dendritic cells

DCs represent a heterogeneous population of cells with different phenotypes and functions and are located in different anatomical locations (Shortman and Liu, 2002). In humans, they develop from bone marrow derived CD34⁺ hematopoietic cells and differentiate along different pathways into myeloid or plasmacytoid (lymphoid) DC (Ardavin *et al.*, 2001; Banchereau *et al.*, 2000). The myeloid subset are phenotypically defined as CD14⁻ or CD14_{low}⁺, CD11c⁺, CD1a⁺ cells and include the monocyte-derived interstitial and Langerhans type DCs (Liu *et al.*, 2001). Of particular importance in PMDD are the Langerhans cells. These cells are defined by the expression of Langerin (Henri *et al.*, 2001) and are involved in the presentation of antigens that enter the body through the skin (Lappin *et al.*, 1996; Romani *et al.*, 2001). The plasmacytoid subset is found in the thymus, blood and tonsils and is characterised by expression of CD123, CD45RA and absence of the CD11c marker (Cella *et al.*, 1999). Human myeloid and plasmacytoid are also referred to as DC1 and DC2 since they were once believed to direct Th1 and Th2 differentiation, respectively (Rissoan *et al.*, 1999). Both subsets express high levels of MHC class II and are thus considered potent antigen presenting cells (Mellman *et al.*, 1998). Similar DC subsets exist in the mouse but the myeloid subset may be further subdivided based on the expression of CD8 α (Kamath *et al.*, 2000). However, this means of delineation requires further clarity as there is evidence suggesting that CD8 α is a marker of maturation status and not lineage (Martinez del Hoyo *et al.*, 2002).

1.3.1.1. Dendritic cell activation, maturation and migration

In the non-lymphoid tissues, DCs predominately exist in the immature form where they are highly phagocytic but are inefficient in their antigen processing and T cell activation ability (Cella *et al.*, 1997). They are activated by exogenous and endogenous danger signals such as pathogen related products or inflammatory cytokines (Ulevitch, 2000; Schnurr *et al.*,

2000). For example, DCs express a wide range of TLRs (see Section 1.2.1) which when bound to their cognate ligands signal cell maturation (Akira, 2003). Immature DC expresses other receptors such as macrophage receptor with collagenous structure (MARCO) and DEC205 (CD205) (Swiggard *et al.*, 1995; Kraal *et al.*, 2000) which interact with microbially derived structures. In this respect, DCs form a vital link between innate and adaptive immunity. The source of the pathogenic stimulus impacts on the type of resulting response, with *E.coli* derived LPS (interacts with TLR4) inducing a Th1-type response while *S.aureus* derived peptidoglycan (interacts with TLR2) provoking a Th2-type response (Pulendran *et al.*, 2001; Re and Strominger, 2001). Bystander cells resident at the site of infection also influence the resulting response. NK cells secrete IFN- γ which promotes Th1 differentiation via IL-12 production in maturing DC while IL-10 released by macrophages can lead to the suppression of IL-12 and results in Th2 differentiation (Mailliard *et al.*, 2003; Moore *et al.*, 2001).

Immature DC moving into sites of infection are characterised by expression of a number of chemokines receptors including CCR2 and CCR6 (which bind to monocyte chemotactic protein (MCP) and macrophage inflammatory protein (MIP-3 α), respectively (Vanbervliet *et al.*, 2002). They also express CCR1, CCR5 and CXCR1 which bind to number of chemokines released by macrophages after activation with microbial products such as MIP-1 α , CCL5 and IL-8 (Dieu *et al.*, 1998; Stumbles *et al.*, 2001; Sallusto *et al.*, 1998). Subsequent encounters with antigen lead to down regulation of the inflammatory chemokines receptors and a down regulation of phagocytic activity (Cella *et al.*, 1997). During the course of maturation, the DCs become more responsive to chemokines produced in the secondary lymphoid tissue such as CCL19 and CCL21 (Saeki *et al.*, 1999) as well as up-regulating cell surface expression of CCR7 which mediates homing of T cells to the secondary lymphoid organs (Willmann *et al.*, 1998). The maturation process and the subsequent migration through the afferent lymphatics to the T cell areas of the local lymphoid tissue brings about an increase in cell surface expression of co-stimulatory molecules CD80 and CD86 as well as up-regulation of MHC class I and II which fine tunes the DC for antigen processing and presentation (Banchereau *et al.*, 2000).

1.3.1.2. Antigen processing pathways

Once resident in the paracortical regions of the draining lymph node, the primary responsibility of DCs is the activation of the appropriate response. This is determined in part by the route of antigenic processing within the DC and consequently, by the context in which the resulting peptides are presented. For MHC I restricted presentation, biosynthesised protein is primarily degraded into smaller peptides by the proteasome which are transported by the antigen associated with antigen processing (TAP) into the lumen of the endoplasmic reticulum (ER) (Lankat-Buttgereit *et al.*, 2002). There, TAP associates with the MHC I- β_2 dimer through interactions with the ER resident chaperones calnexin, calreticulin and tapasin (Cresswell *et al.*, 1999) and the stable class I complex is transferred to the cell surface via the Golgi. Class II peptide presentation is predominately associated with peptides derived from exogenous proteins acquired by endocytosis or from internalised plasma membrane proteins (Watts, 2004). MHC II α and β dimers combine with invariant chain (Ii) in the rough ER and pass through the Golgi to endosomal and lysosomal compartments. The Ii is degraded by several proteolytic enzymes of the cathepsin family (Villadangos *et al.*, 1999), allowing the MHC class II molecules to bind to antigenic peptides and get transported to the plasma membrane.

An alternative mechanism for class I antigenic presentation is cross-presentation (Bevan, 1976). The two main intracellular pathways involved in cross-presentation result in ER or endocytic peptide loading and are TAP dependent and independent, respectively (Yewdell *et al.*, 1999). The TAP dependent pathway appears to be the dominant route in DC (Regnault *et al.*, 1999; Norbury *et al.*, 1997) while TAP independent cross-presentation has been reported in macrophages (Castellino *et al.*, 2000). In addition to class I and II presentation, DC express CD1 proteins which are capable of presenting antigen to T cells (Porcelli and Modlin, 1999). Surface expressed CD1 molecules are internalised and directed to the endocytic pathway where they associate with glycolipidic antigens of endogenous and exogenous origin (Jayawardena-Wolf and Bendelac, 2001). CD1 molecules resident on the DC cell surface are also capable of antigen association (Briken *et al.*, 2000).

1.3.1.3. Lymphocyte activation begins at the immunological synapse

Before T cell activation occurs, naïve T cells interrogate the surface of the DC for the MHC/peptide complex specific for its TcR. This interrogation requires the assembly of clusters of receptors on both cell types in very close proximity at an interaction site referred to as the immunological synapse (IS) (see Figure 1). This is a dynamically active structure characterised by a central region of interaction called the central supramolecular activating complex (c-SMAC) surrounded by a peripheral zone or p-SMAC (Monks *et al.*, 1998). The components of the IS are dependent on the synaptic partners involved but typically, the c-SMAC consists of one or more clusters of CD3/TcR/co-receptor-MHC/peptide and CD28-CD80/CD86 complexes while a number of integrins and their ligands such as lymphocyte function associated molecule-1 (LFA-1)-intracellular adhesion molecule 1 (ICAM-1) occupy the p-SMAC (Grakoui *et al.*, 1999). Larger molecules such as the sialoglycoprotein CD43 and the protein tyrosine phosphatase CD45 (both important for lymphocyte differentiation) are sterically excluded from the central activation zone (Leupin *et al.*, 2000; Roumeir *et al.*, 2001). The duration of the synapse is also dependent on the interaction partners and can last for many hours (~10-12) or just minutes (~2-20) for Th and CTL cells, respectively (Iezzi *et al.*, 1998; Stinchcombe *et al.*, 2001b).

Intuitively, one would expect that the primary function of the IS is to provide a suitable environment for TcR signalling and T cell activation. However, its exact function is unresolved. There is evidence suggesting that it is not involved in initiating TcR signalling with TcR signalling events happening before mature IS formation (Lee *et al.*, 2002). Postulated alternative roles include the polarisation of cytokines specific to the type of activated T cell (Stinchcombe *et al.*, 2001a), facilitating the transfer of APC derived MHC-peptide complexes to CTL (Huang *et al.*, 1999) and in the case of DC-T cell interaction, conditioning the T cell for activation upon recognition of the appropriate MHC/peptide complex. The latter idea is supported by the finding that DC is the only immunologic cell type that can maintain the IS in the absence of specific antigen (Revy *et al.*, 2001).

Initially, association of TcR/CD3, CD4/CD8 and CD28 with their respective binding partners is accompanied by internal cytoskeletal activity such as the movement of the microtubule organising centre to a position close to the synapse in the activating T cell (Kupfer *et al.*, 1991) and in the DC (Al-Alwan *et al.*, 2003). Rearrangement of the T cell cytoskeleton facilitates recruitment of further TcR/CD3 and CD28 complexes into the synapse from other areas on the cell surface (Moss *et al.*, 2002). Cell to cell dialogue instructs the DC to direct recently processed antigen to the antigen specific IS reinforcing the original stimulus and maintaining the synaptic structure (Chow *et al.*, 2002). CD28 ligation contributes to T cell activation by lowering the threshold of stimulated TcR required for a given response (Viola and Lanzavecchia, 1996) and by converting a weak MHC/peptide signal into a strong one (Manickasingham *et al.*, 1998). Furthermore, it is important for stimulating IL-2 and IL-12 receptor expression (Schweitzer *et al.*, 1998). Other receptors are recruited to the IS at later time-points such as negative regulator of activation, cytotoxic T lymphocyte associated antigen-4 (CTLA-4) (Carreno *et al.*, 2000) and members of the TNF receptor family such as OX40 (CD134), 4-1BB (CD137) and CD27 (Paterson *et al.*, 1987; Kwon *et al.*, 1989; Bigler *et al.*, 1988, respectively). In particular, the induction of OX40 and 4-1BB on T cells after synaptic formation (~24 hours) corresponds with their primary functions of maintaining cell division initially regulated by CD28 and preventing cell death in the late primary response of activated CD4+ and CD8+ T cells, respectively (Rogers *et al.*, 2001; Cooper *et al.*, 2002).

1.3.1.4. Initiation of T cell signalling

Ligation of the TcR activates the lymphocyte-specific protein tyrosine kinase p56Lck which phosphorylates specific residues within immunoreceptor-based tyrosine activation motifs (ITAMs) on CD3 and TCR ζ chains (Sunder-Plassmann *et al.*, 1997). This results in the phosphorylation and recruitment of zeta associated protein-70 (ZAP-70) to the TcR/Lck complex (Weiss and Littman, 1994) where it subsequently phosphorylates a number of adaptor proteins such as linker for activation of T-cell signalling (LAT) and SH2-domain-containing leukocyte protein of 76 kDa (SLP-76) amongst others (Jordan *et al.*, 2003). The end result is calcium mobilisation which activates several transcription factors including

nuclear factor of activated T cells (NF-AT) (Rooney *et al.*, 1994) and NF- κ B (Jmieson *et al.*, 1991). These signalling pathways result in the activation of genes required for T-cell proliferation and differentiation as well as the synthesis of important cytokines and chemokines (Kuo and Leiden, 1999). In addition to these shared signalling pathways, type specific transcription factors are activated such as T-bet and GATA binding protein-3 (GATA-3) which play a role in the onset of Th1 and Th2 type immunity, respectively (Szabo *et al.*, 2000; Zheng and Flavell, 1997). Furthermore, thymopoeisis studies have identified that duration of signalling events also serve to differentiate between various T cell subsets (Yasutomo *et al.*, 2000). The differentiation of naïve T cells into CD8+ and CD4+ effectors is represented in Figure 1.

1.3.2. Activation of CD8+ T cells

Once activated, CD8+ naïve lymphocytes develop into effector CTL and central and effector memory cells. The pathways of differentiation that create these populations are unclear with evidence for both “linear” (antigen induced differentiation of naïve T cells into cytotoxic effectors followed by survival of a specific subset to form memory cells) and “branched” (antigen induced differentiation leads to the separate induction of effector and pre-memory lineages) differentiation models (Kaeche *et al.*, 2002a).

1.3.2.1. Tc1 and Tc2 CD8+ effectors

CD8+ effectors are characterised by their ability to induce target cell apoptosis via specific effector mechanisms (i.e. effector CTL) and/or cytokine secretion (Figure 1). During a vigorous response to virus, a large effector response is established (Whitmire *et al.*, 2000). Several cell divisions are required (>8-10) before a naïve CD8+ T cell can become a functionally active CTL with each division cycle occurring approximately every 6-8 hrs (Opferman *et al.*, 1999). The two main pathways responsible for effector activity in functional CTL are the perforin dependent granule exocytosis and the Fas ligand (FasL)/Fas pathway (Russell and Ley, 2002). In the former mechanism, perforin and granzymes stored in lysosomal secretory granules are secreted at the centre of the immunological synapse in a

process regulated by the GTP binding protein rab27a (Stinchcombe *et al.*, 2001a). Death of the target cell requires perforin dependent plasma membrane permeabilization followed by granzyme A and B proteolysis of key cytoplasmic proteins (Beresford *et al.*, 1999; Darmon *et al.*, 1995). For the latter, TcR ligation results in FasL expression on the surface of CTL which bind to target cell expressed Fas molecules and activate enzymes that lead to apoptosis of the infected cell by destruction of its structural cytoskeleton proteins and by chromosomal degradation (Sharma *et al.*, 2000). Analogous to CD4⁺ T cells, CD8⁺ effector subsets may be characterised by their cytokine secretion patterns. These include Tc1 cells which are defined by production of IFN- γ and Tc2 cells which predominately secrete IL-4 and IL-5 (Mosmann *et al.*, 1997). Naïve CD8⁺ cells exhibit a preference for Tc1 differentiation especially in the presence of IL-12 and IFN- γ . However, Tc2 development can occur when IL-4 is available in large amounts (Sad *et al.*, 1995; Noble *et al.*, 2001; Croft *et al.*, 1994). The latter population provides B cell help albeit at lower levels than that provided by Th2 cells (Maggi *et al.*, 1994; Cronin *et al.*, 1995). In humans, CD8⁺ effectors may also be defined by CD45RA expression coupled with the absence of CD27 expression (Hamann *et al.*, 1997).

1.3.2.2. Linear or branched differentiation?

Two different models are used to explain the relationship between CD8⁺ effector and memory populations. The most popular hypotheses is the linear model in which naïve T cells, in response to antigen differentiate into CD8⁺ effectors followed by a subsequent progression of a specific subset into memory cells (Kaeche *et al.*, 2002b). By following the transcriptional and functional profile of effector and memory cells, Kaeche and colleagues (2002a) obtained heterogeneous gene expression patterns demonstrating a dynamic and progressive shift from an effector phenotype toward the memory phenotype over the course of an immune response. The progression of CD8⁺ effectors into memory cells is reflected in the observation that both populations have a similar spectrum of TcR specificities coupled with similar patterns of cytokine secretion (Liu *et al.*, 1997). These data are further supported by findings of Jacob and Baltimore (1999), Opferman *et al.* (1999) and Wherry *et al.* (2003) all of whom suggest memory development as the product of a progressive

differentiation programme. In contrast, others have demonstrated that CD8+CD62L_{low} and CD8+CD62L_{high} CD45RO+ memory populations were dissimilar suggesting that effector and memory populations are independently derived and are not inter-convertible (Baron *et al.*, 2003).

1.3.2.3. CD8+ T_{CM} and/or T_{EM} phenotypes

Following the effector phase, a memory CD8+ population forms which is maintained in the absence of antigen (Lau *et al.*, 1994; Murali-krishna *et al.*, 1999). Typically, these cells are divided into central memory (T_{CM}) and effector memory (T_{EM}) cells, characterised by the differential expression of CCR7 and different homing molecules as well as presence or absence of effector function (Sallusto *et al.*, 1999). T_{CM} cells predominately reside in the lymphoid tissues and are CCR7+CD62L_{High} while T_{EM} cells circulate in non-lymphoid tissues, have lost expression of CCR7 and are heterogeneous for CD62L expression. In humans, memory is additionally distinguished by expression of CD45RA with T_{CM} and T_{EM} cells expressing low and no levels of this cell surface marker, respectively (Urbani *et al.*, 2002). There is evidence for and against T_{CM} effector function in both human (Hislop *et al.*, 2001; Rakov *et al.*, 2003) and in mouse (Unsoeld *et al.*, 2002; Masopust *et al.*, 2001). In contrast, human and murine T_{EM} cells exhibit consistent levels of cytotoxic effector activity (Hamann *et al.*, 1997; Sallusto *et al.*, 1999). The latter memory subset expresses adhesion molecules and chemokine receptors which target these cells to specific tissues. These include cutaneous associated lymphocyte antigen (CLA) and CCR4 which direct T_{EM} cells to the skin (Campbell *et al.*, 1999) and $\alpha 4\beta 7$ and CCR9 (Zabel *et al.*, 1999) which identify gut homing T_{EM} cells.

Whether the T_{CM} and T_{EM} subsets are mutually exclusive memory subpopulations is unclear. Data from a murine LCMV model suggest a reciprocal conversion between subsets which was dependent on the physiological conditions encountered (Wherry *et al.*, 2003) while others report that T_{EM} cells are the end product of memory (Champagne *et al.*, 2001; Migueles *et al.*, 2002). In apparent contradiction, analysis of CD8+ T cells in human

peripheral blood led Baron *et al.* (2003) to suggest that T_{CM} and T_{EM} are largely independent subpopulations.

1.3.2.4. Regulating differentiation and maintenance of memory

There are a number of factors which determine the fate of CD8⁺ memory cells including those which regulate the differentiation of CTL into memory and those which maintain the memory subpopulations. CD4⁺ help through CD40/CD40L ligation is important for promoting differentiation into memory cells (Bennett *et al.*, 1998; Whitmire *et al.*, 1996; Johansen *et al.*, 2004) and maintaining memory (Cardin *et al.*, 1996; Janssen *et al.*, 2003). The pro-survival proteins B-cell lymphoma 2/3 (bcl-3, bcl-2) have been implicated as possible regulators of a prolonged effector response and in the induction of memory (Grayson *et al.*, 2000; Mitchell *et al.*, 2001). The generation of effectors and maintenance of memory are also influenced by various factors including the strength (i.e. antigen concentration) and duration of TcR signalling (Tanchot *et al.*, 1997; Iezzi *et al.*, 1998), low DC/T cell ratios during activation (Langenkamp *et al.*, 2002) and co-stimulatory molecules like 4-1BB (Diehl *et al.*, 2002; Shedlock *et al.*, 2003). Cytokines such as IL-7 are extremely important survival factors for memory CD8⁺ cells (Goldrath *et al.*, 2002; Schluns *et al.*, 2000) while IL-15 is critical for survival and proliferation of these cells (Fehniger *et al.*, 2001; Ku *et al.*, 2000; Schluns *et al.*, 2002). Furthermore, IL-21 has been implicated in the induction of the T_{CM} phenotype (Eberl *et al.*, 2002). Finally, as a negative regulation mechanism of memory, IFN- α mediated cell death of existing CD8⁺ memory cells can occur when new CTL responses of different antigen specificity emerge (Varga *et al.*, 2001; McNally *et al.*, 2001).

1.3.3. Activation of CD4⁺ T cells

Presentation of peptide-MHC II complexes to naïve CD4⁺ T cells by APCs triggers a cell proliferation and differentiation programme leading to the development of either Th1 or Th2 effectors, defined on the basis of the cytokines they produce and by their immunomodulatory behaviour (Murphy *et al.*, 2000). The Th1 phenotype predominately produces

IFN- γ but also secretes lymphotoxin and is a key component of cell-mediated immunity (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 which mediate B cell immunity and antibody production (O'Garra, 1998; Romagnani, 1994). Typecasting the effector response in this way may be an oversimplification however as others have demonstrated heterogeneity of effector CD4⁺ cells even within polarised effector populations (Kelso *et al.*, 1999; Openshaw *et al.*, 1995). This heterogeneity is a result of the complex interactions of different cytokines (O'Garra, 1998), cell-signalling proteins (Murphy *et al.*, 2000) and transcription factor and chromatin remodelling events (Agarwal and Rao, 1998) which occur during the activation process. Proliferation is also influenced by other factors including the type and maturity of the APC (Cella *et al.*, 2000), antigen dose, (Ruedl *et al.*, 2000), presence of co-stimulatory molecules (Corry *et al.*, 1994) and the requirement for DNA replication (Richter *et al.*, 1999; Laouar and Crispe, 2000). Within a population of activated CD4⁺ cells, a certain proportion secretes IL-2 and possesses the ability to differentiate into either Th1 or Th2 depending on the conditions of subsequent stimulation (Th0) (Openshaw *et al.*, 1995). Others have mRNA expression of markers denoting Th1 or Th2 cells and are committed to either lineage after subsequent stimulation while some cells can secrete Th1/Th2 specific cytokines immediately after activation (Wu *et al.*, 2002; Wang and Mosmann, 2001; Lanzavecchia and Sallusto, 2000). Currently, there is an ongoing debate on whether naïve CD4⁺ cells are instructed or selected to mature into polarised Th1 or Th2 effector cells (Reiner and Seder 1999). Here, the CD4⁺ helper response is discussed in terms of the classical Th1/Th2 paradigm.

1.3.3.1. Th1 commitment

Th1 cells produce IFN- γ and play an important role in cell mediated immunity by activating macrophages to release pro-inflammatory cytokines and to eliminate intracellular pathogens (Bogdan *et al.*, 1991). The central cytokine associated with Th1 cell development is IL-12 (Manetti *et al.*, 1993). It is a heterodimeric cytokine composed of p35 and p40 subunits (Trinchieri and Scott, 1999) and is produced by a wide range of DC after appropriate stimulation (e.g. interaction of myeloid DC expressed TLR2 and TLR4 with

peptidoglycan and LPS, respectively) (Boonstra *et al.*, 2003). It binds to the IL-12 β 1 and IL-12 β 2 receptor on CD4⁺ T cells and activates the Signal Transducer and Activator of Transcription (STAT) pathways (Rogge *et al.*, 1997; Szabo *et al.*, 1995). Indeed, expression of the IL-12 β 2 receptor is considered a marker for Th1 differentiation as Th2 cells do not express this receptor due to direct activity of IL-4 (Sinigaglia *et al.*, 1999). Mice deficient in IL-12 (Magram *et al.*, 1996) and STAT-4 (Thierfelder *et al.*, 1996) have substantially reduced IFN- γ production and Th1 responses. IL-18 augments the activity of IL-12 in Th1 development and can work together with IL-12 to induce IFN- γ production in differentiating and effector Th1 cells. Indeed in fully committed Th1 cells, no TcR-CD4 activation signal is required when IL-12/IL-18 are present (Robinson *et al.*, 1997).

Other important cytokines include the type 1 IFNs which play a role in promoting Th1 differentiation by working together with TcR activation (Nguyen *et al.*, 2002) and IFN- γ itself which up-regulates the Th1 transcription factor, T-bet which in turn activates IL-12 β 2 and IFN- γ (Afkarian *et al.*, 2002). More recently, IL-23 and IL-27 have been implicated in Th1 development (Oppmann *et al.*, 2000; Pflanz *et al.*, 2002). IL-23 preferentially induces IFN- γ production and proliferation of the memory subset of T cells (Belladonna *et al.*, 2002). IL-27 induces naïve CD4⁺ T cells to proliferate into Th1 cells in an IL-12 independent manner and also synergises with IL-12 to activate IFN- γ production and Th1 polarisation (Pflanz *et al.*, 2002). In contrast, Th1 development is negatively influenced by IL-10 (Fiorentino *et al.*, 1989) by inhibiting APC maturation (Buelens *et al.*, 1997) and IL-12 production (Murphy *et al.*, 1994). Th1 immunity is also influenced by chemokines such as CXCR3 and CCR5 (Qin *et al.*, 1998) as well as adhesion molecules such as E- and P-selectin (Syrbe *et al.*, 1999; Austrup *et al.*, 1997).

At a transcriptional level, T-bet is the critical transcription factor linked with Th1 development (Szabo *et al.*, 2000). It is up-regulated via the STAT-1 pathway which is activated through the IFN- γ receptor and operates independently of the IL-12 induced STAT-4 pathway (Mullen *et al.*, 2001). Ectopic expression of T-bet in STAT^{-/-} mice can induce IFN- γ production in cells cultured under Th2 conditions (Mullen *et al.*, 2001).

Furthermore, CD4⁺ T cells from T-bet^{-/-} mice have severe defects in Th1 polarising ability (Szabo *et al.*, 2002). Expression of T-bet induces DNase I hypersensitivity in the IFN- γ gene locus suggesting a role in epigenetic Th1 regulation (Mullen *et al.*, 2001). The STAT-4 pathway is also important in Th1 immunity operating in an IL-12 induced manner (Bacon *et al.*, 1995) possibly by activating directly the IFN- γ promoter (Xu *et al.*, 1996). In contrast, GATA-3 inhibits the development of Th1 cells by down-regulation of the IL-12 β 2 receptor (Ouyang *et al.*, 1998).

1.3.3.2. Th2 commitment

Th2 immunity is characterised by production of its signature cytokine IL-4 and is associated with the humoral immune response (Bird *et al.*, 1998). These cells also secrete IL-5, IL-6, IL-9, IL-10 and IL-13 (Abbas *et al.*, 1996). IL-4 plays a central role in the development of Th2 phenotype and naïve CD4⁺ T cells stimulated by TcR activation in the presence of IL-4 leads to Th2 effectors capable of producing IL-4, IL-5 and IL-13 (O'Garra, 1998). It also suppresses IFN- γ production from Th1 cells (Hsieh *et al.*, 1992). It binds to one of two different types of IL-4 receptors, both of which contain the IL-4R α subunit (Lai *et al.*, 1996). Other important cytokines include IL-6 which induces Th2 cell differentiation by up-regulation of IL-4 while suppressing Th1 development via the suppressor of cytokine signalling-1 (SOCS1), a STAT-1 phosphorylation inhibitor (Diehl *et al.*, 2000) and IL-11 which promotes IL-4 and IL-5 production and inhibits IL-12 release from macrophages (Curti *et al.*, 2001). Additionally, IL-21 is preferentially expressed on Th2 cells and also serves to inhibit IFN- γ production in Th1 cells (Wurster *et al.*, 2002). Th2 type immunity may also characterised by expression of the chemokine receptor CCR3 which is regulated by IL-4 and IL-13 and leads to production of eotaxin by endothelial and epithelial cells in response to allergic inflammation (Li *et al.*, 1999). Other chemokine receptors preferentially expressed on Th2 cells are CCR4 and CCR8 (Randolph *et al.*, 1999; Sozzani *et al.*, 1998).

Several Th2 related transcription factors have been identified. The most prominent ones include GATA-3, STAT-6 and c-MAf (Zheng and Flavell, 1997). Of these, GATA-3 is

considered the master regulator of the Th2 phenotype. Indeed, ectopic expression of GATA-3 under Th1 polarising conditions can cause Th2 development while down regulating IFN- γ production (Ferber *et al.*, 1999). Furthermore, expression of this transcription factor in fully differentiated Th1 clones results in IL-4 and IL-5 production (Ouyang *et al.*, 2000). A number of GATA-3 binding sites have been identified on the IL-4 and IL-13 genes indicating that it plays an important role in Th2 specific epigenetic regulation (Takemoto *et al.*, 1998). It has the capacity to induce other Th2 transcription factors such as c-MAF as well as auto-inducing further GATA-3 production (Ouyang *et al.*, 2000) and it down regulates the IL-12 β 2 receptor using an IL-4 independent mechanism (Ouyang *et al.*, 1998).

1.3.3.3. Fate of CD4+ effector cells

Memory CD4+ T cells display many functional differences to the naïve and effector cells from which they are generated including induction by lower amounts of antigen, rapid secretion of increased levels of cytokines, decreased susceptibility to activation-induced cell death and persistence in the absence of class II re-stimulation (Swain, 2000). Whether the transition from effector to memory happens through a linear differentiation pathway is unclear. Th2 effectors give rise to memory cells without further differentiation supporting a linear default pathway from effector to memory (Hu *et al.*, 2001). This is also supported by the finding that the phenotype of polarised memory cells reflects the phenotype of adoptively transferred polarised effectors (Swain *et al.* 1999). In contrast, results from Wu *et al.* (2002) suggest that Th1 effectors may not follow a similar differentiation pathway. Within the memory population itself, there is evidence for progressive differentiation from T_{CM} to T_{EM} with increased signal strength suggesting that both memory subpopulations are inter-related (van de Merwe, 2001). Furthermore, memory CD4+ cells formed from polarised Th1 and Th2 effectors can alter their cytokine profile after re-stimulation suggesting a degree of flexibility in the memory phase (Messi *et al.*, 2003). CD4+ memory

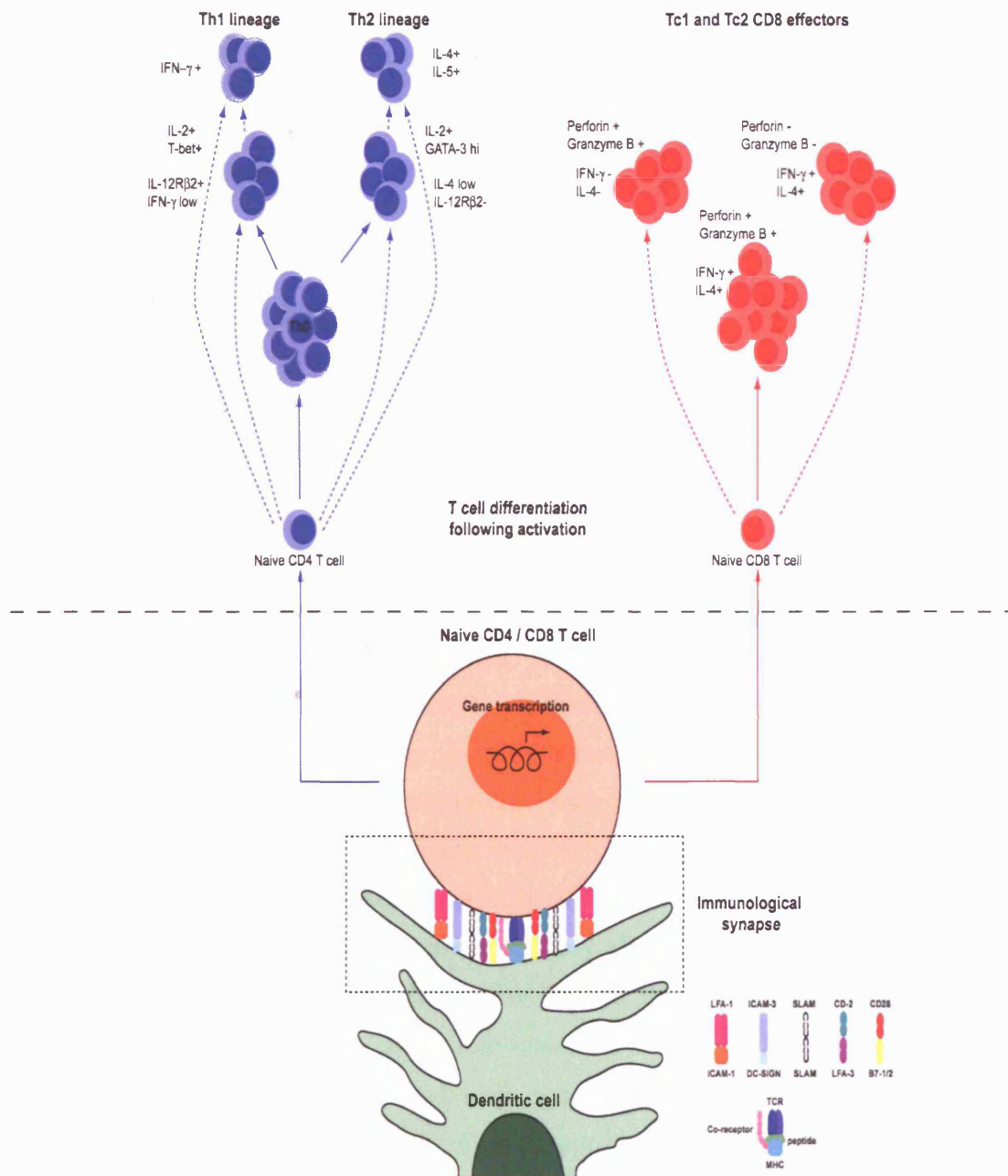


Figure 1.1. Schematic of the receptor-ligand interactions which form the immunological synapse and CD4+/CD8+ T cell differentiation after activation.

cells are maintained by IL-7 (Seddon *et al.*, 2003) but unlike CD8⁺ memory T cells, they do not respond to IL-15 (Zhang *et al.*, 1998).

1.3.4. Regulatory T cells

Specific T cell populations with regulatory/suppressive functions also exist *in vivo* and these cells are involved in important immune functions such as self-tolerance, anti-tumour immunity and control of infection (Sakaguchi, 2000; Shevach, 2002). Fundamentally, regulatory T cells (T_{REG}) may be divided into naturally occurring CD4⁺ CD25⁺ T cells and induced T_{REG} cells (Bluestone and Abbas, 2003). CD4⁺ CD25⁺ cells are selected in the thymus in response to intermediate levels of TcR signalling from MHC-II-antigenic peptide complexes (Jordan *et al.*, 2001). They may be characterised by low expression of CD45RB as well as OX40, L-selectin (CD62L) and glucocorticoid-induced TNF receptor family-related gene (GITR) (McGuirk and Mills, 2002; McHugh *et al.*, 2002; Lepault and Gagnerault, 2000; Shimizu *et al.*, 2002, respectively). However, as these cell surface molecules are not exclusive to T_{REG} cells, these subsets are more accurately defined by the expression of the transcription factor FoxP3 (Fontenot *et al.*, 2003). Once activated by antigen, T_{REG} cells mediate suppression in a cell contact, antigen independent manner leading to inhibition of IL-2 production in the target cell (CD4⁺CD25⁻ T cells or DC) (Thornton and Shevach, 1998). The second category of T_{REG} cells are referred to as induced T_{REG} cells and may be further subdivided into Tr1 and Th3 cells on the basis of IL-10 and TGF- β production, respectively (Roncarolo *et al.*, 2001; Weiner, 2001). They are derived from normal CD4⁺ CD25⁻ cells and may represent an altered state of differentiation (Bluestone and Abbas, 2003). Both subsets suppress Th1 and Th2 responses in a cell independent, antigen non-specific, cytokine mediated manner (Weiner, 2001; Groux, 2003). Both naturally occurring and induced T_{REG} cells cooperate *in vivo* and there is evidence that suggests that sub-populations of the naturally occurring CD4⁺CD25⁺ T_{REG} cells have the ability to induce Tr1 or Th3 T_{REG} cells of different antigen specificity (Jonuleit *et al.*, 2002; Dieckmann *et al.*, 2002).

1.3.5. CD8-CD4 interactions

1.3.5.1. T cell co-operation

Synergistic interactions between antigen specific CD8+ and CD4+ responses are required for control of many viruses (Cardin *et al.*, 1996; Matloubian *et al.*, 1994). Indeed, in many viral infections, there is a reliance on CD4+ T cells for the development of the CD8+ response (Jennings *et al.*, 1991; Zajac *et al.*, 1998). In instances where CTL precursor frequencies are high (Mintern *et al.*, 2002) or when viral infection leads to direct up-regulation of co-stimulatory molecules such as B7-1/B7-2 on dendritic cells (Wu and Liu, 1994), CD4+ cells are not required for initial activation of the CD8+ response. However, optimal generation, differentiation and maintenance of memory CD8+ T cells requires the presence of CD4+ T cells during the initial CTL activation phase (Riberdy *et al.*, 2000; Belz *et al.*, 2002; Johansen *et al.*, 2004). The CD4+ response provides help by secretion of cytokines such as IL-2 which promote the expansion of the CD8+ response (Matloubian *et al.*, 1994). A second mechanism of help involves activation of DCs by ligation of CD4+ T cell expressed CD40L with DC expressed CD40 (CD4-APC-CD8 pathway). This in turn leads to up-regulation of co-stimulatory molecules such as B7-1/B7-2 licensing the DC to induce CD8+ T cell activation through CD40 ligation (Schoenberger *et al.*, 1998; Ridge *et al.*, 1998) and to secrete cytokines important to CTL induction such as IL-12 (Curtsinger *et al.*, 1999). There is also evidence for direct CD4-CD8 T cell help as activated CD8+ T cells transiently express CD40 after activation (Bourgeois *et al.*, 2002).

Using mathematical modelling, Wodarz and Jansen (2001) postulate a role for both mechanisms of help with each mechanism showing prominence at different stages of viral infection. They argue that in the primary phase of infection when virus load is high, the quickest and most efficient way to activate the CD8+ response is firstly the generation of CD8+ self-help through production of autocrine IL-2 and after a number of cell divisions, the secretion of IL-2 by CD4+ helpers (Deeths *et al.*, 1999). Subsequent phases of infection may favour the CD4-APC-CD8 pathway which promotes viral clearance at low virus loads. Other investigators have also demonstrated CD8-CD4 (Zhong *et al.*, 2001), CD8-CD8

(Sherritt *et al.*, 2000) and CD4-CD4 (Gerloni *et al.*, 2000) T cell co-operation. From a DNA vaccination perspective, there is much evidence for co-operative CD4-CD8 T cell interactions (Maecker *et al.*, 1998; Kursar *et al.*, 2002; Ren *et al.*, 2003). Furthermore, Creusot and colleagues demonstrated co-operation between CD4⁺ T cells of different specificity following PMDD (2003a; 2003b). Consistent with the view of Behrens *et al.* (2004) that linked recognition is a requirement for the provision of help, Creusot *et al.* observed that the CD4-CD4 helper effect was greatest when both antigens were delivered to the same dendritic cell.

1.3.5.2. T cell competition

There is also much evidence for competition between T cells. Competition between CD8⁺ T cells of same or different specificity (Probst *et al.*, 2002; Palmowski *et al.*, 2002; Lawson *et al.*, 2001; Grossmann *et al.*, 2001) has been most commonly observed although CD4-CD4 T cell competition has also been noted (Smith *et al.*, 2000). For CD8⁺ T cells, it is generally agreed that intraclonal competition (i.e. antigen specific competition) occurs (Kedl *et al.*, 2000; Wolpert *et al.*, 1998; Sandberg *et al.*, 1998). These reports suggest that competition for antigen occurs at the level of the APC and that the competitive effects may be alleviated by increasing the number of available APCs (Butz *et al.*, 1998a; Grufman *et al.*, 1999a). Other factors that contribute to intraclonal competition are high precursor frequency and TcR affinity for antigen. Support for the former stems from the observations that competition is most prevalent following a number of immunisations (Kedl *et al.*, 2002; Palmowski *et al.*, 2002) and that pre-immunity to a subdominant antigen reduces the response to a more dominant antigen in subsequent immunisations against both antigens (Chen *et al.*, 2000c). The link between TcR affinity for antigen and T cell competition was demonstrated by Kedl *et al.* (2002) who transferred high and low affinity anti-ovalbumin CD8⁺ T cells to a previously primed host and observed that the high affinity T cells out-competed the lower affinity T cells after antigenic challenge. Some groups have suggested that high affinity T cells compete by APC killing (Grufman *et al.*, 1999b; Matloubian *et al.*, 1999; Ludewig *et al.*, 2001) which would serve to reduce access to antigen by lower

affinity T cells. Others implicate an antigen extraction mechanism whereby high affinity T cells induce antigen loss from the surface of the APC (Kedl *et al.* 2002).

Whether interclonal T cell competition occurs is less definite as there is evidence for (Wolpert *et al.*, 1998; Kedl *et al.*, 2000) and against (Smith *et al.*, 2000) this phenomenon. Indeed, the findings of Probst *et al.* (2002) led to the suggestion that interclonal competition does not play a functionally important role in the development of T cell responses. However, where it has occurred, there is a requirement for presentation of both antigens on the same APC (Sandberg *et al.*, 1998). This type of competition is much less efficient than antigen-specific competition (Kedl *et al.*, 2000). Ge *et al.* (2004) observed that CD8⁺ T cells with different TcRs compete only for cytokine factors surrounding the DC-T cell cluster such as IL-7 and IL-15. Furthermore, Rodriguez *et al.* (2002) showed that CD8⁺ T cells against an immunodominant epitope inhibit responses to subdominant epitopes by the suppressive effects of IFN- γ secreted by the immunodominant T cell population.

The co-operative and competitive interactions between T cells of different specificities (i.e. following PMDD of influenza nucleoprotein and Ovalbumin encoding plasmid DNA) are investigated further in chapters 6 and 7.

1.4. DNA vaccination

The ability of the adaptive immune system to develop memory is the foundation for the successful application of vaccines in modern medicine. Over the past century, many vaccines strategies have been so successful that infectious diseases such as smallpox, polio and diphtheria have either been totally eliminated or are on the verge of extinction. Development of these “conventional” vaccines centred on the whole disease-causing organism itself, either in the attenuated or killed form. Other standard approaches used to confer protective immunity included using sub-cellular units of organisms (sub-unit vaccines) and inactivated toxins from these organisms. Further developments in recombinant DNA technology and molecular biology saw the use of attenuated bacteria and virus vectors for gene delivery, but pre-existing immunity to these vectors dominated the immune response to the delivered antigen. Issues surrounding vaccine preparation, safety and pre-existing immunity together with the onset of diseases such as HIV and cancer which prove somewhat refractory to conventional vaccine strategies have precipitated the requirement for alternative approaches to vaccine development. One of these approaches is DNA vaccination.

DNA vaccination (also referred to as nucleic acid vaccination) is a vaccination method whereby the host is inoculated with a eukaryotic expression plasmid encoding antigen (Ulmer *et al.*, 1993). Interest in this area was first initiated when Dubensky *et al.* (1984) demonstrated replication of viral and recombinant plasmid DNA in mouse liver and spleen. The findings of Wolff *et al.* (1990) who reported that β -galactosidase protein expression was obtained following injection of naked plasmid DNA into mouse and Tang *et al.* (1992) who demonstrated that gene gun vaccination (PMDD) successfully stimulated antibody production to the encoded gene product suggested DNA vaccination as a viable alternative to conventional vaccination. The simplicity of the composition of plasmid DNA has many immunological advantages over recombinant and viral vaccines. Once delivered *in vivo*, plasmid DNA becomes transcriptionally active, harnessing the cells cytoplasmic machinery to produce antigen. Unlike immunisation with protein, intracellular synthesis of the

encoded antigen enables it to fold in its native conformation with normal post-translational modifications closely mimicking natural infection (Chattergoon *et al.*, 1997).

Absence of an associated viral coat allows it to escape the presence of neutralising antibodies and eliminates a potentially immunodominant response to coat surface proteins (Robertson and Griffiths, 2001). Furthermore, recombinant viruses risk mutation or reversion to more pathogenic forms and may suffer from pre-existing immunity (Restifo and Rosenberg, 1999). Studies addressing the risks associated with DNA inoculation have shown safe and well tolerated administration with no local or systemic reactions and absence of anti-DNA antibodies (Boyer *et al.*, 1999; MacGregor *et al.*, 1998). In other studies, mice immunised with DNA developed anti-DNA antibodies (Griffiths, 1995). However, antibody titres in that particular study were so low that they failed to induce auto-immunity. Kanellos *et al.* (1999) confirmed immunity without initiation of chromosome integration and nucleic acid auto-immunity. Moreover, the frequency of integration of foreign DNA into the host chromosome was estimated to be much lower than spontaneous mutation (Nichols *et al.*, 1995).

DNA vaccination encompasses a wide range of delivery mechanisms including electroporation, jet-injection and liposomes (Clark and Johnson, 2001). The most widely used methods include needle injection of naked DNA into the muscle (Wolff *et al.*, 1990) or skin (Raz *et al.*, 1994) and PMDD to the skin (Williams *et al.*, 1991; Torres *et al.*, 1997). Less invasive means of introducing DNA involve topical application of plasmid to the skin or mucosal surfaces. Examples of these are intranasal (Klavinskis *et al.*, 1999), oral (Etchart *et al.*, 1997) and intravaginal (Wang *et al.*, 1997) administration all of which lead to the induction of antigen specific responses. DNA vaccination by PMDD is mainly focused on here.

1.4.1. Particle-mediated DNA delivery

Particle-mediated DNA delivery is a technique that uses compressed helium gas to accelerate microscopic beads coated with DNA into the skin. This method is very effective

at delivering plasmid DNA to the cells of the epidermis and requires only nanogram quantities of DNA (Pertmer *et al.*, 1995). Since it was first used to deliver DNA into plant cells (Klein *et al.*, 1987), it has found applications in many different areas including gene therapy (Yang *et al.*, 2004) and DNA vaccine delivery (Fuller *et al.*, 2002). The potential of PMDD as a vaccination tool was first highlighted by Tang *et al.* (1992) who observed antigen specific antibody responses in serum of inoculated mice following intra-dermal DNA administration. Because PMDD is dependent on the physical nature of the delivery process, it has found a logical application in epidermal delivery (Haynes *et al.*, 1996). Additionally, the epidermal layers of the skin are home to the myeloid DC, the Langerhans cells. These professional APC which are present in the skin at a density of 500-1000 cells/mm² (Chen and Payne, 2002) present a valuable target for plasmid DNA delivered by PMDD providing ready access to the adaptive immune response of the host.

1.4.1.1. Intra-dermal injection versus gene gun

DNA vaccines are most commonly delivered to the dermal and epidermal layers of the skin by needle injection or by PMDD. Comparative analysis of the two methods indicates that PMDD is more effective than intra-dermal needle injection as it possesses the ability to elicit equivalent CTL and antibody responses with substantially less DNA (Pertmer *et al.*, 1995; Degano *et al.*, 1998). Indeed, Pertmer and colleagues demonstrated that only 16 ng DNA/immunisation was required to elicit significant antibody titres and CTL activity, 5000 fold less than the amount needed if the DNA was administered by needle injection. Furthermore, the optimal doses of DNA eliciting an immune response in mice for intra-dermal needle injection is ~10-100 µg compared with ~0.1-1 µg for PMDD. For humans, ~200-2500 and ~1-4 µg of DNA are required to generate immune responses using DNA injection and PMDD, respectively (Payne *et al.*, 2002).

This difference may be because intra-dermal injection delivers the DNA to the extracellular space in the skin where it is susceptible to degradation by nucleases (Levy *et al.*, 1996). Alternatively, the differential requirements for DNA in both methods may also be due to the “danger signal” phenomenon which is important in stimulating the innate response

(Porgador *et al.*, 1998). The damage caused by the physical impact of gold particles delivered at high pressure may function as a danger signal that effectively recruits and activated DCs (Culley and Olszewska, 2003). Porgador *et al.* suggested that the danger signal effect was responsible for the influx of 20-30,000 non-transfected CD11c+ DCs in the local draining lymph node 24 hours after PMDD. This represented a two fold increase in the number of DC normally resident there. Furthermore, they demonstrated that PMDD of gold particles alone resulted in an increase in DC trafficking to the local draining lymph node suggesting the adjuvant effect of ballistic bombardment itself. Additionally, one important advantage of PMDD over intra-dermal DNA injection is that it provides a simple way of introducing multiple genes and adjuvants such as GM-CSF (Xiang and Ertl, 1995) to the same antigen presenting cell, thereby improving the quality and quantity of the resulting immune response.

1.4.1.2. Gene gun modulates type 1/2 immunity?

The ability of a DNA vaccine delivered by PMDD to elicit a Th1 or Th2 response is dependent on a number of factors including delivery mode, nature of antigen synthesised, and immunisation regimen used (i.e. number and time intervals between immunisations). Some of the earlier studies suggest that gene gun mode of delivery preferentially elicits a Th2 response characterised by the production of IL-4, IL-5 and an IgG1 antibody response. In contrast, administration of naked DNA (especially intramuscular inoculation) leads to a predominantly Th1 response (Feltquate *et al.*, 1997; Pertmer *et al.*, 1996). However, more recent data argues against this with Yoshida *et al.* (2000) and Frelin *et al.* (2003) demonstrating strong CTL responses and type 1 immunity against the respective antigens. Furthermore, Fuller and colleagues (2002) demonstrated significant CTL activity and a low antibody response against SIV antigens in rhesus macaques following multiple immunisations by PMDD. Whether the synthesised antigen is secreted or retained in the cytoplasm may also influence the nature of the resulting response. Torres *et al.* (1999) reported predominately IgG1 antibody responses to influenza and measles virus hemagglutinins (HAs) following PMDD delivery. Increasing the time intervals between successive immunisations resulted in increased type 1 activity (characterised by IFN- γ

secretion) against HIV-1 gp120 in mice and rhesus macaques using PMDD (Prayaga *et al.*, 1997; Fuller *et al.*, 1996, respectively).

1.4.1.3. Plasmid design

A DNA plasmid in its simplest form usually consists of a bacterial plasmid that contains a gene-encoding antigen and poly-adenylation sequence under the control of a strong eukaryotic promoter. An antibiotic resistance gene and a bacterial origin of replication are also included for amplification in bacteria (Gurunathan *et al.*, 2000). One of the more important factors to consider when generating such plasmids is the optimisation of plasmid design in order to generate plasmids which produce more antigen and are more stimulatory to the host immune system. To this end, a number of different promoters, enhancers and poly-adenylation sequences have been tested in DNA vaccines (Kwissa *et al.*, 2000; Xu *et al.*, 2001). Investigations from Lee *et al.* (1997) using the chloramphenicol acetyltransferase (CAT) gene as an indicator demonstrated that, of several different promoters, the cytomegalovirus immediate early enhancer-promoter (CMV-IE) was the most effective at inducing antibody responses following intra-muscular DNA plasmid inoculation. These findings were supported by Zhang and colleagues (2002) who reported a similar outcome in human prostate cancer cell lines transfected by PMDD. Others however have reported down regulation of antigen production *in vivo* in vectors containing viral promoters such as CMV due to inhibitory effects of cytokines such as IFN- γ and TNF- α (Qin *et al.*, 1997). Where there is a requirement for localised antigen production, promoters such as the desmin promoter (Kwissa *et al.*, 2000) or the creatine kinase promoter (Gebhard *et al.*, 2000) which specifically target expression in muscle cells may be employed. Likewise, the dectin-2 promoter (Takashima and Morita, 1999) or the scavenger receptor gene promoter (Xiang *et al.*, 1997) is preferentially active in DC.

Further expression optimisation is achieved through insertion of the “Kozak” consensus sequence in front of the start codon of the opening reading frame (Kozak, 1987) as well as through mammalian codon optimisation techniques. Indeed, Nagata *et al.*, (1999) demonstrated that codon optimisation correlated well with protein production levels in

mammalian cells. Additional regulatory elements shown to enhance antigen expression include intron A (Niwa *et al.*, 1990; Chapman *et al.*, 1991) and exon-1 (Simari *et al.*, 1998). The beneficial effects of intron A are attributed to increased levels of RNA polyadenylation (Huang and Gorman, 1990) or by acting as an enhancer (Buchman and Berg, 1988). The latter two components are compared for their effect on driving antigen expression in chapter 5.

An important attribute of plasmid DNA is its ability to stimulate the innate response through immuno-stimulatory sequences (ISSs). These sequences are present in the backbone of bacterial plasmids in the form of cytidine-phosphate-guanosine (CpG) motifs (Krieg *et al.*, 1995). Because bacterial DNA has a much higher level of unmethylated CpG motifs compared with mammalian DNA, it possesses the ability to stimulate the mammalian innate immune system (Krieg *et al.*, 1996). It binds to TLR9 which is expressed in the endosomal compartment of DCs and B cells (Ahmad-Nejad *et al.*, 2002) and causes these cells to produce pro-inflammatory cytokines such as IL-12 and IFN- γ , promoting Th1 development (Klinman *et al.*, 1996). As such, it behaves as an adjuvant to the adaptive immune response. Indeed, ISSs have been successfully used to improve the efficacy of DNA vaccines when present in the bacterial plasmid backbone (Sato *et al.*, 1996) or when co-delivered as an adjuvant (Cho *et al.*, 2000). However, this adjuvant effect is species specific and so optimisation is required for each intended host system (Ballas *et al.*, 1996).

1.4.1.4. Mechanisms of antigen presentation

Following delivery, DNA vaccines engage both MHC I and II antigen processing pathways provoking cellular and humoral immunity, respectively (Chattergoon *et al.*, 1997). There is much evidence supporting direct transfection of DC following DNA vaccination (Raz *et al.*, 1994; Timares *et al.*, 2003). Using electron microscopic analysis and fluorescence microscopy, Condon *et al.* (1996) demonstrated the presence of LCs containing 1 μ m gold beads expressing green fluorescent protein in draining lymph nodes after PMDD indicating direct DC transfection. Iwasaki and colleagues (1997) showed that the induction of a CTL

response was restricted to the MHC haplotype of transfected bone marrow derived DC and not to the haplotype of transfected somatic cells following PMDD. Skin ablation and grafting experiments demonstrated that T and B cell activation occurred in mice whose vaccination sites remained intact for 24 hrs after PMDD (Klinman *et al.*, 1998). This together with the finding that skin grafts transferred to naïve recipients within that time interval were capable of launching a response in those animals suggested that immunity was induced by cells which migrated quickly from the vaccination site to the draining lymph node. Further support for direct DC transfection as the predominant mechanism of antigen presentation was provided by Timares *et al.* (2003) who used a drug inducible system to switch on transgene expression within transferred LCs in recipient mice. Upon induction, antigen synthesised within the transferred LCs led to a cellular response which was equal in magnitude to that observed in the donor animals after vaccination. In contrast, the humoral response was 10-fold lower.

From a quantitative perspective, Porgador *et al.* (1998) attempted to elucidate the number of directly transfected DC following PMDD. They demonstrated that after five intra-dermal gene gun immunisations, only 50-100 directly transfected DC were recovered from an individual draining lymph node. However more recently, Garg and colleagues (2003) who used a sensitive genetic tagging technique observed that this number was ~100 fold higher than that calculated by Porgador and colleagues.

Synthesised antigen may also access the class I pathway through cross presentation (Bevan, 1976). Support for the contribution of this presentation pathway to the development of CD8+ immunity following PMDD comes from the studies of Cho *et al.* (2001). Using a tissue specific promoter system, significant CD8+ responses were obtained as a result of non-APC specific antigen expression. MHC II restricted presentation of antigen plays an important role in the induction of the humoral response following PMDD (Torres *et al.*, 1997). Further support for this antigen presentation pathway comes from Loirat and colleagues (1999) who demonstrated that antigen produced exclusively in muscle myocytes under the control of the muscle specific desmin gene promoter resulted in a qualitatively and quantitatively similar cellular response to that obtained with a non-specific CMV

promoter was used. DCs can take up secreted vaccine antigen expressed in transfected bystander cells such as skin keratinocytes or myocytes. In this context, these somatic cells act as antigen depots supplying small amounts of secreted antigen and contribute to the induction and maintenance of the antibody response (Eisenbraun *et al.*, 1993; Torres *et al.*, 1997). It is likely that direct DC transfection, cross-presentation and MHC II presentation play important roles both individually and collectively in the induction of protective immunity following gene gun immunisation.

1.4.1.5. Enhancing the efficacy of DNA vaccines

A large number of approaches have been employed to enhance the potency of DNA vaccines. Some of these strategies have been tested in PMDD models while others have been investigated following needle injection of DNA. Generally, these approaches may be divided into those which result in improved antigen uptake in DC and DC maturation, increased antigen degradation and presentation and the improvement of the DC-T cell interaction.

As discussed previously, unmethylated CpG motifs present in the plasmid backbone enables targeting of DNA to DC and stimulates maturation by binding to TLR9 (Klinman *et al.*, 1996). Gene gun delivery of genes encoding the apoptosis inducing proteins, murine caspase 2 and a chimera of murine 2 prodomain and human caspase 3 enhanced the T cell responses to influenza hemagglutinin by preferentially directing the contents of apoptosed somatic cells to DC (Sasaki *et al.*, 2001; Sasaki *et al.*, 2002). In a similar fashion, Leitner and colleagues (2003) successfully broke tolerance to a tumour antigen by co-delivering a DNA vaccine encoding an alphavirus replicon. This provided a source of double stranded RNA which induced caspase-dependant apoptosis and stimulated the innate response through DC uptake. You *et al.* (2001) showed that a DNA vaccine for hepatitis B antigen e fused to an IgG Fc fragment was efficiently captured and processed by DC while Boyle and colleagues (1998) successfully used CTLA-4 as means to target antigen to DC. Chen *et al.* (2000a) demonstrated that linkage of the gene encoding heat shock protein (HSP)-70 targeted the model antigen (HPV type 16 E7) to DC and increased the CD8+ T cell

response in a CD4⁺ independent manner. An alternative approach to increasing the number of antigen containing DC is the use of protein transduction domains (PTDs) from translocatory proteins. Such proteins include VP22 from herpes simplex virus type 1 (HSV-1) and the HIV protein tat (Prochiantz, 2000). Hung *et al.* (2001a) demonstrated that HSV-1 VP22 enhanced the intracellular spreading of the linked HPV-16 E7 antigen to surrounding cells thereby increasing the E7 specific CTL response. Other strategies used to direct antigen to and mature DC include the delivery of constructs expressing antigen fused to chemokines such as interferon inducible protein 10 (IIP-10) and monocyte chemotactic protein 3 (MCP-3) (Biragyn *et al.*, 1999) and the extra-cellular domain of the cytokine Fms-like tyrosine kinase 3 (Flt-3) (Hung *et al.*, 2001c). Delivery of the cytokine GM-CSF has also proved effective at recruiting and maturing DC (Xiang and Ertl, 1995).

Increasing the level of antigenic degradation and improving the efficiency of presentation within the DC provides another way of increasing vaccine potency. DNA vaccines may be constructed which express only the immuno-dominant epitopes from a targeted antigen. These vaccines, termed minigene or minitope vaccines have been shown to induce CTL efficiently (Uchijima *et al.*, 1998; Blaney *et al.*, 1998). Intracellular targeting strategies to enhance MHC I restricted presentation include addition of leader sequences which localises the target peptide in the ER (Ciemik *et al.*, 1996) and insertion of sequences which increase antigen ubiquitination and degradation (Wu and Kipps, 1997). Additionally, Rodriguez and colleagues (1998) demonstrated that the strategy of antigen ubiquitination results in enhanced CTL immunity. Antigen fused to a protein of foreign origin (i.e. influenza nucleoprotein₃₆₆₋₃₇₄–green fluorescent protein conjugate) resulted in efficient CTL induction through increased CD4⁺ T cell help (Wolkers *et al.*, 2002). Antigens fused to other proteins such as bacterial toxin translocation domains (Hung *et al.*, 2001b), calreticulin (Cheng *et al.*, 2001), centrosomal γ -tubulin (Anton *et al.*, 1999) and heat shock proteins (Trimble *et al.*, 2003) have also been demonstrated to augment the antigen specific CTL response through increased MHC I presentation. Arrington *et al.* (2002) reported a strong enhancement of the CTL response to multiple viral antigens when vectors encoding cholera toxin and *E coli* heat-labile enterotoxin were co-delivered by PMDD.

Equally, many strategies have been used to augment MHC II restricted CD4⁺ T cell responses. DNA vaccines have utilized signals which target the class II processing pathway in the lysosome and late endosome. Ji *et al.* (1999) targeted HPV-16 E7 antigen to these sub-cellular compartments by co-delivery of the gene encoding the lysosome-associated membranous protein-1 (LAMP-1). Likewise, the lysosomal integral membrane protein-II (LIMP-II) was successfully used to induce antigen specific CD4⁺ T cell induction (Rodriguez *et al.*, 2001). Van Tienhoven and colleagues (2001) used an Ii construct (with class II associated invariant chain peptide (CLIP) replaced by a HSP-60 derived epitope) to induce HSP-60 CD4⁺ T cell responses.

In addition to enhanced processing and presentation, improving the quality and the longevity of the DC-T cell interaction is important for increased vaccine potency. This was well illustrated by Kim *et al.* (2003) who used anti-apoptotic factors such as BCL-xL and BCL-2 amongst others to prolong the survival of transfected DC. Although this strategy is in direct contrast with the pro-apoptotic approach adopted by Sasaki and colleagues (2002), it resulted in enhanced antigen specific CD8⁺ T cell responses. Other groups have co-delivered cytokines such as IL-12 and the co-stimulatory molecule B7 which amplified T cell activation and augmented cell mediated and humoral immunity (Kim *et al.*, 1997).

The microenvironment in which the antigen is presented may be manipulated to steer the activity of the antigen specific immune response. Numerous studies have reported that co-administration of vaccine antigen with genetic adjuvants can alter the immune response to the antigen (Maecker *et al.*, 1997; Chow *et al.*, 1998). Inclusion of genes encoding IL-18 (Zhu *et al.*, 2003), IL-2, IL-12 and IL-15 (Moore *et al.*, 2002) increased the magnitude of the response to the antigens of interest. Positive results were obtained in PMDD studies when IL-12 (Prayaga *et al.*, 1997) and IFN- γ (Siegel *et al.*, 2001) were co-delivered.

1.4.1.6. Prime-boost strategies

Because of the relatively small quantity of protein produced *in vivo* following DNA immunisation, this regimen by itself is limited in its ability to elicit a long lasting and potent immune response. To overcome this shortcoming, many groups have adopted heterologous prime-boost strategies whereby the immune system is primed to the target antigen delivered in one vector and boosted by delivery of the same antigen in the presence of a second but different vector (Ramshaw and Ramsey, 2000). In such approaches, DNA appears to be more effective at priming the immune response while a whole range of different viruses are preferentially used for priming. Priming with DNA avoids anti-viral vector host responses (Xiang *et al.*, 1999) while viral boosting provides more antigen and a broader spectrum of antigen related targets (Estcourt *et al.*, 2002). There is much evidence supporting the use of heterologous prime-boosting strategies of which DNA immunisation by PMDD is one component (Fuller *et al.*, 1997a; Fuller *et al.*, 1997b). Regimes in which a DNA prime is followed by a poxvirus boost have proved effective as demonstrated by the findings of Hanke *et al.* (1999) and Degano *et al.* (1999) who, compared with DNA immunisation only, reported increased CTL responses to HIV and malaria epitopes, respectively. Doria-Rose *et al.* (2003) demonstrated protection from CD4+ T cell depletion in a simian-HIV model (SHIV89.6P) using a DNA prime/vaccinia virus boost. Others have demonstrated complete protection against plasmodium berghei sporozoite challenge in mice using a DNA/modified vaccinia virus Ankara (MVA) regime (Degano *et al.*, 1999).

1.4.1.7. Mucosal immunity

As the vast majority of infectious pathogens enter the body through the mucosae, adequate protection requires local immunity. A strong mucosal response is characterised by the presence of antigen specific mucosal CD8+ T cells and secretory IgA antibodies. As such, the challenges facing mucosal and systemic DNA vaccination are different (Lajeunesse *et al.*, 2004). DNA vaccines delivered to mucosal sites by PMDD have demonstrated protective immunity or enhanced protection compared with systemic routes of administration (Loehr *et al.*, 2000; Wang *et al.*, 2003). In most instances, greatest efficacy

was obtained when PMDD was used as the priming component of a heterologous prime-boost regime. Livingston and colleagues (1998) demonstrated protective anti-human growth hormone IgA and IgG antibody responses following PMDD via the vaginal mucosa in mice. Furthermore, delivery of DNA to Peyers patches followed by a systemic boost (i.e. intra-dermal PMDD) in rabbits also resulted in high anti-HIV glycoprotein 120 IgG and IgA responses (Winchell *et al.*, 1998). Although the preponderance of information on heterologous prime-boost strategies indicate better results when DNA is used as the priming component, Eo *et al.* (2001) observed optimal mucosal responses when vaccinia virus was used as the priming vector and DNA administered mucosally was used as boost. Additionally, that mucosal prime-boost strategy also induced good systemic responses.

1.4.1.8. PMDD efficacy in humans

Translating protective and therapeutic activity from mice to humans is the major goal for DNA vaccines. There are a number of studies demonstrating partial protection in non-human primate models (Doria-Rose *et al.*, 2003; Puaux *et al.*, 2004) but very few of these have been tested in clinical settings. As described in section 1.4.1.6, most of these approaches involve a DNA prime and fowlpox virus boost (Degano *et al.*, 1999; Schnedier *et al.*, 1998; Kent *et al.*, 1998). In the first published PMDD human trial, Roy *et al.* (2000) demonstrated total sero-conversion with 12/12 individuals developing protective antibody responses against hepatitis B surface antigen (HBsAg). These antibody responses compared favourably with those obtained in other human studies which employed intra-muscular delivery of much higher amounts of DNA (up to 1000 fold more) (MacGregor *et al.*, 2000; MacGregor *et al.*, 2002). In a separate study, Rottinghaus and colleagues (2003) demonstrated protective immunity against HBsAg in 12/16 individuals, all of whom were previously unresponsive to conventional hepatitis B vaccination. Finally, McConkey *et al.* (2003) investigated the effect of a heterologous prime-boost regime (i.e. PMDD or intra-muscular injection DNA prime/MVA boost) on the induction of protective immunity against pre-erythrocytic malaria thrombospondin-related adhesion protein (TRAP). Although the results indicated partial immunity, the prime-boost regime yielded antigen specific T cell responses that were 10 fold higher than those obtained by either PMDD or

MVA alone. Furthermore, PMDD proved much more potent than intra-muscular injection for priming with several fold higher post boost T cell responses obtained.

1.5. Particle-mediated DNA delivery *in vitro*

The efficacy of transfection of mammalian cell lines *in vitro* is an important aspect to consider in the creation of potent DNA vaccines. This is especially relevant to PMDD where the *in vitro* and *in vivo* transfection processes are similar. Due to this close association, many studies have centred on PMDD as a transfection tool.

PMDD was pioneered by Klein and colleagues (1987) to introduce DNA into monocotyledonous plants. Since it was first used by Zelenin *et al.* (1989) to transfect mammalian cells, it has been employed successfully to transfect a wide variety of isolated tissue explants and the primary cultures derived from these explants as well as a number of cultured mammalian cells (see Table 1.1). It provides a fast and simple method for transfection with a requirement for only small quantities of DNA. Because it depends on the physical process of penetrating the cell membrane, it does not have a necessity for a particular type of receptor or biochemical structure on the cell type been transfected. For this reason, it has been used in cells types such as neuronal cells which have proved refractory to transfection by conventional techniques (Klimaschewski *et al.*, 2002).

Quantitative assessment of PMDD efficiency *in vitro* is varied. The transfection rate is dependent on the type of device used and the origin of cells transfected (Biewenga *et al.*, 1997). Zhang and colleagues (2002) reported transfection rates of up to 35 % in human prostate tumour cell lines while other investigators observed transfection levels of ~1-5 % in murine cell lines (Kitagawa *et al.*, 2003). In some instances, the technique compares well with other transfection techniques with Thompson *et al.* (1993) and Jiao *et al.* (1993) reporting ~5 and 100 fold increases in transfection efficiency compared with other transfection methods, respectively. In other cell lines, the technique does not compare well (Smith *et al.*, 2001). There are a number of ballistic parameters which are important to

Reference	Device	Force (psi)	Cell type	Transfection efficiency (%)
Kitagawa <i>et al.</i> , 2003	Powderject	300	MCA205: mouse fibrosarcoma	4.1
	Accell		NIH-3T3: mouse fibroblast	1
Novakovic <i>et al.</i> , 1999	Bio-Rad Helios	100	B-16: mouse B-16 melanoma	9.6
			MCF-7: human adenocarcinoma	10.5
			L929: mouse fibroblast	3.9
Murphy and Messer, 2001	Bio-Rad PDS-1000	1350	Mouse cerebellum organotypic cultures	34
Schilthuis <i>et al.</i> , 1993	Dupont PDS-1000	450	B1H1: Newt blastemal cells	10
Klimaschewski <i>et al.</i> , 2002	Bio-Rad Helios	120	Rat sympathetic neuron	10
Wellmann <i>et al.</i> , 1999	Bio-Rad Helios	120	PC12: rat pheochromocytoma	> 5
		100	Dog cerebellar granule cells	> 10
Smith <i>et al.</i> , 2001	Bio-Rad Helios	150	Human PBMC derived dendritic cells	0
Timares <i>et al.</i> , 1998	Rumsey-Loomis	950	XS106 DC: mouse LC cell line	0.25
		1000	NS46 FB: mouse fibroblast	2
O'Brien <i>et al.</i> , 2001	Bio-Rad Helios (modified)	175	HeLa: human cervical carcinoma	~ 26-28
			Hek293: human embryo kidney	~ 26-28
Fitzpatrick-McElligott, 1992	Bio-Rad PDS-1000	1300	CHO: chinese hamster ovary	1.5
			ELA: mouse lymphocyte	0.6
Zhong <i>et al.</i> 2002	Bio-Rad Helios	150	DU145, PC-3, LNCaP : all human prostate cell lines	~ 35
Heiser, 1994	Bio-Rad PDS-1000	1100	CHO: chinese hamster ovary	4

Table 1.1: A partial list of cultured cell lines and primary cultures transfected by PMDD.

optimise such as the amount of DNA loaded on gold beads and the acceleration force required for DNA delivery (Heiser, 1994). For the hand held delivery system with the helium propulsion system (e.g. Helios and PowderJect XR1 gene gun devices), the latter parameter appears to be critically important but very variable. Some investigators have reported optimal transfection using delivery pressures as high as 1300 psi (Fitzpatrick-McElligot, 1992) while others have optimised the process using pressures as low as 100 psi (Novakovic *et al.*, 1999). Indeed, many of the parameters involved in transfection have been demonstrated to be inter-dependent (Klein *et al.*, 1987) highlighting the requirement for process optimisation for the combination of each device used and each cell line transfected.

An important application of PMDD is the transfection of DCs, either *ex vivo* or *in vitro*. For human DCs, transfection efficiency appears to be dependent on DC precursor type. DCs which develop from bone marrow derived CD34+ hematopoietic cells and which divide in culture have been shown to be resistant to transfection by PMDD (Lundqvist *et al.*, 2002). For non-dividing monocyte derived DC, there are both successful and unsuccessful transfection reports (Tuting *et al.*, 1998; Smith *et al.*, 2001, respectively). To date, successful transfection of murine DC has not been reported. The efficiency of PMDD to a number of cell lines, including somatic and murine bone marrow derived DC is explored in chapters 3 and 4.

1.6. Introduction to this PhD project

This research project has two main aims. The first is to explore the relationships between the efficiency of PMDD when used to stimulate immune responses *in vitro* with the more usual *in vivo* models. The development of *in vitro* surrogate models which can, at least in part, replace *in vivo* testing is becoming increasingly important in many areas of pharmaceutical research. In addition, these studies may identify some of the critical parameters which ultimately regulate and limit the overall *in vivo* immune response. Thus the first main aim is the development of an *in vitro* system featuring gene gun as the means of plasmid delivery. A number of cell lines are investigated for their capacity to become transfected by PMDD including a number of cultured mammalian cells lines as well as more immunologically relevant cell types like bone marrow derived DCs. Using this *in vitro* system, various different aspects of PMDD technology are examined. The effect of parameters such as immunising dose and the effect of single versus dual plasmid immunisation on the resulting immune response are investigated. Additionally, other facets of this technology which contribute to overall efficacy *in vivo* including the role of intracellular competition and intracellular targeting are addressed.

In the second part, the responses obtained by co-immunising with two independent antigens delivered together by PMDD are investigated in more detail. Following co-immunisation, the immune responses to both antigens may interact negatively (i.e. compete) or positively (i.e. co-operate) with each other. The final immune response reflects the balance between these opposing immune forces. The relative contribution of antigenic competition and co-operation on the resulting immune response and the mechanisms involved are investigated. The effect of pre-existing immunity, antigen dose and antigen linkage are examined.

Chapter 2

Material and Methods

2.1. Mice

BALB/c and C57BL/6 mice were obtained from Charles River U.K. RAG1/F5 mice (C57BL/10 background, H-2^b haplotype), transgenic for TcR to nucleoprotein of influenza A/NT/60/68 residues 366-374 (Mamalaki *et al.*, 1993) were obtained from the National Institute of Medical Research, Mill Hill, London, U.K. DO.11.10 mice (BALB/c background, H-2^d haplotype), transgenic for TcR to chicken ovalbumin peptide residues 323-339 (Murphy *et al.*, 1990), were obtained from Dr Ken Murphy (Washington University School of medicine, St. Louis, MO, U.S.A.). Colonies of DO.11.10 and RAG1/F5 mice were kept at Bury Green Farm, GSK, U.K. All breeding, husbandry and experiments were carried out in accordance with U.K. animal experimental legislation.

2.2. Cell lines

The RAW and J774 macrophage cell lines and the EL4 T cell lymphoma cell line were cultured in complete RPMI 1640 (i.e. supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 x 10⁻⁵ M β-mercaptoethanol). The DAP-3/ICAM cell line is a mouse fibroblast cell line stably transfected with I-Ad and ICAM-1. This was cultured in complete IMDM containing the selection markers for ICAM-1 and I-Ad, 666 µg/ml geneticin and HAT (3.3 x 10⁻⁵ M hypoxanthine, 3 x 10⁻⁵ M aminopterin and 5.33 x 10⁻⁵ M thymidine) (all Sigma), respectively. The MC57 fibrosarcoma cell line was cultured in DMEM supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. All tissue culture reagents were purchased from Gibco BRL unless otherwise stated. All cell lines were obtained from GSK, U.K. and tested negative for *Mycoplasma* contamination.

2.3. Plasmids

2.3.1. Reporter gene studies

Plasmids pGL3-CMV-luciferase (Promega), pCIneo-CMV- β -GFP and pCIneo-CMV- β -galactosidase (Invitrogen) were used in reporter gene studies.

2.3.2. pVAC1 vectors

All pVAC1 vectors (Table 2.1, vector with no insert; see Figure 2.1) were kindly supplied by Dr Ian Catchpole, GSK, U.K. pVAC1 vectors are derived from the pCI vector (Promega), contain the CMV immediate early promoter, intron A, the SV40 polyadenylation sequence and the ampicillin resistance gene. The pVAC1.OVAc vector encoding cytoplasmically localised chicken ovalbumin protein was made by cutting the *HindIII* cDNA insert of pVAC1.OVAs vector with *SstI* (method by Tabe *et al.*, 1984).

2.3.3. p7313.NT plasmid synthesis

Four p7313.NT plasmids (encoding nucleoprotein of influenza strain A/NT/60/68) with different promoter configurations were constructed (Figure 2.2). The p7313 vectors and the pspC1 vector were kindly supplied by Dr Peter Ertl, GSK, U.K. The p7313 vectors contain the rabbit globin polyadenylation sequence and the kanamycin resistance gene. The pspC1 vector was used as the source of the minimal CMV promoter in the synthesis of the p7313.m.NT plasmid.

2.3.3.1. NT amplification

The NT gene fragment was amplified from pVAC1.NT by PCR using PWO polymerase with primer optimisation kit (Roche Diagnostics). Each reaction contained 0.5 μ l pVAC1.NT template DNA, 0.5 μ l dNTPs (dATP, dCTP, dGTP, dTTP, each at 100 mM, Gibco BRL), 0.125 μ l of primers; 5'-

GAATTCGCGGCCGCGATGGCGTCCCAAGGCACCAAA-3' and 5'-GAATTCATATGTTACTCGAGATTGTCGTACTCCTCTG-3' (MWG-Biotech AG), 0.25 μ l of PWO enzyme, 16.5 μ l of DNase and RNase free water (Sigma) and 2 μ l of PCR optimisation buffer. The reaction was carried out as follows: 60 sec at 95°C, 40 cycles (30 sec at 95°C, 30 sec at 45°C, 120 sec at 72°C), 120 sec at 72°C. Optimal pH and MgSO₄ conditions were 8.3 and 1.5 mM, respectively. The amplified gene fragment was purified using the QIAquick PCR purification protocol (Qiagen) and checked for correct gene sequence.

2.3.3.2. p7313.plc.NT plasmid synthesis

The NT gene fragment was ligated with the p7313.plc vector. Firstly, both p7313.plc (vector) and NT (insert) were cut with NotI and NdeI (New England Biolabs) for 1 hour at 37°C. The reaction containing the vector consisted of 2 μ l of DNA, 2 μ l each of NotI and NdeI, 4 μ l of 10 x REact 3 buffer (Gibco BRL) and 30 μ l water. The reaction containing the insert consisted of 30 μ l of DNA, 2 μ l each of NotI and NdeI, 4 μ l of 10 x REact 3 buffer and 2 μ l water. The vector and insert were run on a 0.8 % agarose gel (Sigma) containing 0.5 μ g/ml Ethidium Bromide (Sigma) in TBE buffer for 20 min at 100 volts, excised and purified using the gel purification cleanup protocol (Qiagen). The vector was cut again with NotI and NdeI for 1 hour at 37 °C. The reaction contained 5 μ l of 10 x REact 3 buffer, 2 μ l each of NotI and NdeI and 41 μ l of DNA (from gel purification cleanup). The vector was purified using the QIAquick PCR purification protocol and treated with SAP (Roche diagnostics). This reaction contained 1 μ l of SAP, 5 μ l of 10 x REact 3 buffer and 45 μ l of vector (from PCR purification cleanup). The reaction was carried out as follows: 1 hour at 37°C followed by 20 min at 65°C. The ligation was performed for 10 min at RT using 1 μ l of vector, 3 μ l of insert, 4 μ l of 2 x ligase buffer and 0.8 μ l of ligase (both from Ligafast rapid ligation kit, Promega).

The ligation product (p7313.plc.NT) was transformed into JM109 competent cells (Promega) as follows: 2.5 μ l of ligation product was added to 25 μ l of cells and kept on

Vector name	Vector size (base pairs)	Coding antigen	CD8 T cell Epitope analysed	CD4 T cell Epitope analysed	Genbank accession reference
pVAC1.NT	6138	Nucleoprotein of influenza A/NT/60/68	D ^b restricted NT ₃₆₆₋₃₇₄ sequence ASNENMDAM	I-A ^b restricted NT ₄₁₃₋₄₃₅ sequence SVQRNLPFDKPTIMAAF TGNTG	J02137
pVAC1.OVAs	6502	Chicken ovalbumin secreted	K ^b restricted OVA ₂₅₇₋₂₆₄ sequence SIINFEKL	I-A ^d restricted OVA ₃₂₃₋₃₃₉ sequence ISQAVHAAHAEINEAGR	V00383
pVAC1.OVAc	6124	Chicken ovalbumin non-secreted	K ^b restricted OVA ₂₅₇₋₂₆₄ sequence SIINFEKL	I-A ^d restricted OVA ₃₂₃₋₃₃₉ sequence ISQAVHAAHAEINEAGR	V00383

Table 2.1. pVAC1 vectors used and CD8/CD4 epitopes analysed in this study.

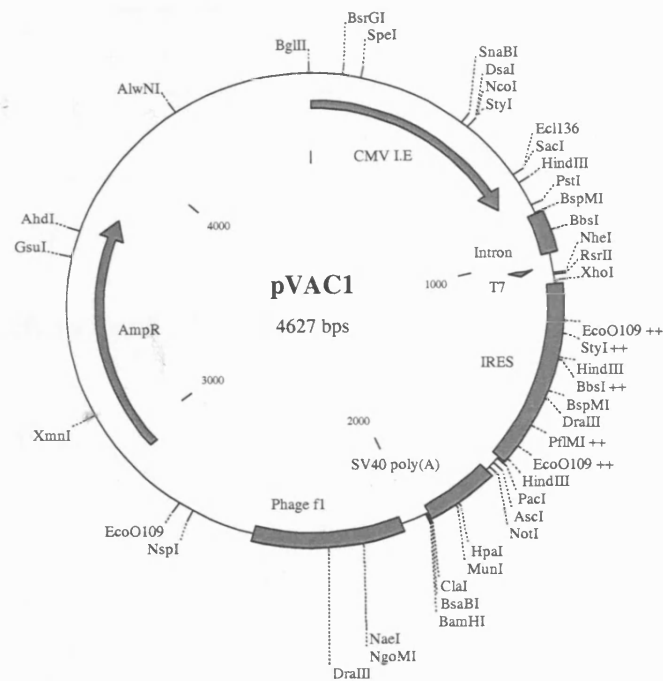


Figure 2.1. Plasmid map of pVAC1.

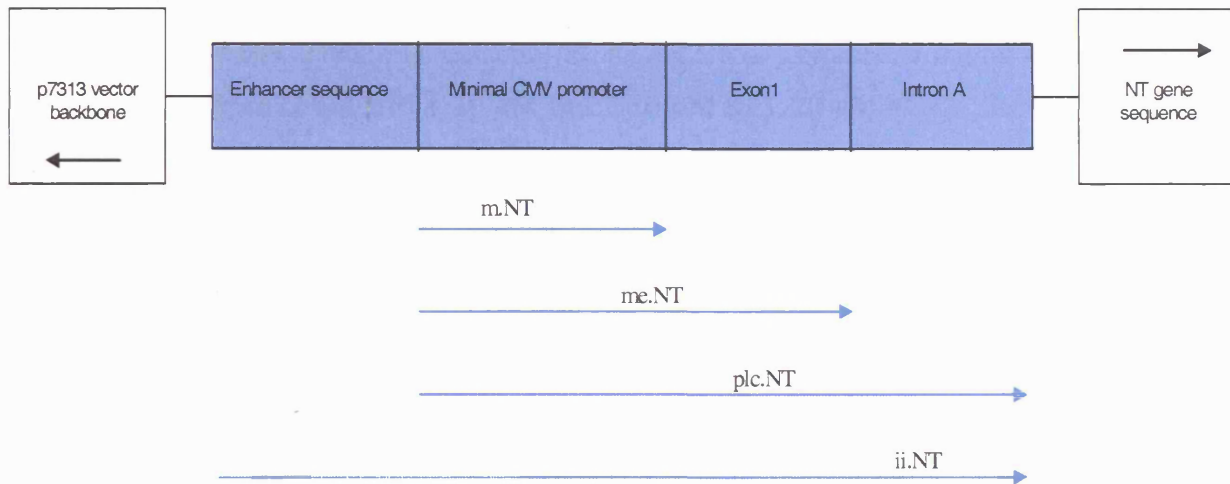


Figure 2.2. Schematic of different p7313.NT promoter configurations used.

ice for 15 min. The cells were heat shocked for 45 sec at 42°C and then placed on ice for 1 min. 100 μ l of SOC medium (containing 20 mg/ml Bacto Tryptone, 5 mg/ml Bacto Yeast Extract (both from Gibco BRL), 10 μ M NaCl, 10 μ M KCl, 20 μ M MgCl₂, 20 μ M MgSO₄ and 20 μ M Glucose (all from Sigma)) was added and the cells incubated at 37°C for 30 min. The cells were plated on 2 x YT plates (8 mg/ml Bacto Tryptone, 5 mg/ml Bacto Yeast Extract and 5 mg/ml NaCl) containing 25 μ g/ml kanamycin (Sigma) and incubated at 37°C. Forty eight hours later, colonies were picked and transferred to universals containing 5 ml of 2 x YT broth with 25 μ g/ml of kanamycin and incubated overnight at 37°C. The plasmid was prepared using Endofree Maxiprep kits (Qiagen). Correct sequence insertion was checked by Bam HI (New England Biolabs) restriction digest as follows: 1 μ l of plasmid was incubated together with 0.5 μ l of Bam HI, 1 μ l of 10 x REact 3 buffer and 7.5 μ l of water for 3 hours at 37°C and then run on a 0.8 % agarose gel containing 0.5 μ g/ml Ethidium Bromide in TBE buffer for 20 min at 100 volts.

2.3.3.3. Construction of p7313.NT plasmids with different promoters

Plasmids p7313.m.NT, p7313.me.NT and p7313.ii.NT were constructed by cutting vectors p7313.plc, p7313.me, p7313.ie (p7313.ii vector – Intron A), p7313.ii, pspCI and plasmid p7313.plc.NT with different combinations of NotI, NdeI and Sse8387 (New England Biolabs) and ligating combinations of isolated DNA fragments (Figure 2.3).

Restriction enzyme digestion was done in two stages. Firstly, NotI and NdeI digestion was performed for 3 hours at 37°C. For reactions where both NotI and NdeI were used, the vectors were incubated for 1 hour with NotI and then NdeI was added for a further two hours. Concentration of plasmid or vector used was ~0.5 μ g/ μ l except for p7313.ii and pspCI which were ~1 and 2 μ g/ μ l, respectively. Next, vectors that required digestion with Sse8387 were purified using the QIAquick PCR purification protocol. Sse8387 digestion was performed for 2 hours at 37°C using 10 x REact 6 buffer (Gibco BRL). Details of the NotI/NdeI and Sse8387 reactions are shown in Figure 2.3. Reaction products were run on a 0.8 % agarose gel containing 0.5 μ g/ml Ethidium Bromide in TBE buffer for 20 min at 100

	p7313.plc.NT	p7313.me	p7313.ie	p7313.ii	pspC1
Restriction enzymes used	NotI / NdeI	NotI / NdeI	NdeI / Sse8387	NotI / Sse8387	NotI / Sse8387
NotI / reaction (μ l)	1.5	1.5	-	1.5	1.5
NdeI / reaction (μ l)	1.5	1.5	1.5	-	-
Water (μ l)	31	31	32.5	33.5	34.0
10 x Rx3 buffer / reaction (μ l)	4	4	4	4	4
DNA / reaction (μ l)	2	2	2	1	0.5
Sse8387 / reaction (μ l)	-	-	1.5	1.5	1.5
BSA / reaction (μ l)	-	-	5	5	5
10 x Rx6 buffer / reaction (μ l)	-	-	5	5	5
DNA / reaction (μ l)	-	-	38.5	38.5	38.5
DNA fragment isolated (bp)	A: 1512	B: 3017	C: 2774	D: 1455	E: 124
DNA fragment description	NT insert	Vector backbone + me promoter	Vector backbone	ii promoter	m promoter

Figure 2.3. DNA fragments (pink fill) isolated for ligations following NotI, NdeI (yellow fill) and Sse8387 (blue fill) enzyme digestion.

volts, excised and purified using the gel purification cleanup protocol.

p7313.m.NT was constructed by ligating 1 μ l of fragment A, 2 μ l of fragment C and 1 μ l of fragment E, p7313.me.NT by ligating 2 μ l each of fragments A and B and p7313.ii.NT by ligating 1 μ l of fragment A with 1.5 μ l each of fragments C and D (Figure 2.3, pink fill). Each ligation contained 4 μ l of 2 x ligase buffer and 0.8 μ l of ligase and was performed for 2 hours at RT. Transformations and plasmid preparation were done as described before. Each plasmid was checked for correct sequence insertion by Bam HI and ClaI (New England Biolabs) restriction digest as follows: 1 μ l of plasmid was incubated together with 0.3 μ l of Bam HI, 0.3 μ l of ClaI, 1 μ l of 10 x REact 3 buffer and 7.4 μ l of water for 1 hour at 37°C and then run on a 0.8 % agarose gel containing 0.5 μ g/ml Ethidium Bromide in TBE buffer for 20 min at 100 volts.

2.4. Peptides and Proteins

Peptides used in *in vitro* and *in vivo* assays were purchased from Genemed Synthesis, Inc., CA, U.S.A. or Washington Singer Labs. The following sequences were obtained: OVA₃₂₃₋₃₃₉, sequence ISQAVHAAHAEINEAGR, OVA₂₅₇₋₂₆₄, sequence SIINFEKL, OVA₂₆₅₋₂₈₀, sequence TEWTSSNVMEERKIKV, nucleoprotein of influenza A/NT/60/68₃₆₆₋₃₇₄ sequence ASNENMDAM and nucleoprotein of influenza A/NT/60/68₄₁₃₋₄₃₅, sequence SVQRNLPFDKPTIMAAFTGNTEG. Ovalbumin protein was obtained from Sigma and the HPV 6 E1 and HIV polymerase proteins were kindly supplied by Dr Karen Barber and Dr Fiona Cook, GSK, U.K. respectively.

2.5. DNA cartridge preparation for particle-mediated DNA delivery

2.5.1. Standard method

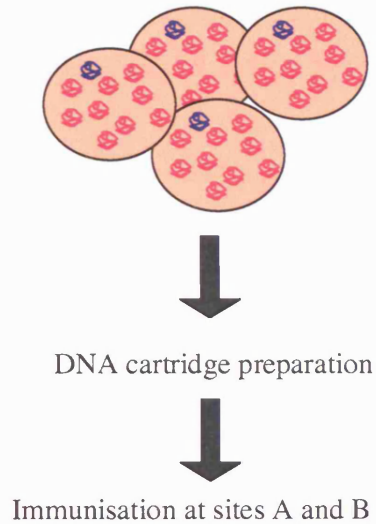
DNA cartridge preparation was based on a method described previously (Eisenbraun *et al.*, 1993; Pertmer *et al.*, 1995). Two μ m diameter gold particles (PowderJect Pharmaceuticals, Madison, Wisconsin, U.S.A.) were sonicated in the presence of 0.05 M spermidine (Sigma)

twice for 10 sec. Plasmid DNA (~1000 ng/ml) was added to achieve the standard DLR of 2000 ng/mg of gold. The DNA was precipitated on to the gold particles by the addition of 1 M CaCl_2 (American Pharmaceutical Partners Inc., L.A., CA, U.S.A.). The DNA/gold complex was incubated for 10 min at RT, washed 3 times in absolute ethanol (previously dried on molecular sieve 3A, BDH) and resuspended in absolute ethanol containing 0.05 mg/ml polyvinylpyrrolidone (Sigma). Tefzel tubing (PowderJect) which had previously been dried with N_2 was placed inside a tube turner (PowderJect) and the DNA coated gold slurry was applied to the inner surface of the tubing by centrifugal force. The tubing was cut into 12.5 mm lengths and stored with dessicant at 4°C. Cartridge DLR was assessed by elution of 2 cartridges/batch in 50 μl DNase and RNase free water for 30 min at 37°C and 5% CO_2 followed by spectrophotometric quantitation using a Genequant II (Pharmacia Biotech). Preparations that contained $500 \text{ ng} \pm 100 \text{ ng}$ DNA/cartridge were considered suitable for use.

2.5.2. DNA cartridges containing two plasmids

In experiments comparing delivery of two plasmids to the same or different immunisation sites, the term “linked” DNA preparation refers to the presence of relevant plasmids on the same gold particles, in the same cartridge and delivered to the same immunisation site. The term “unlinked” DNA preparation refers to the presence of the relevant plasmids on different gold particles, in different cartridges and delivered to different immunisation sites. The linked DNA preparation was made by mixing pVAC1.NT and pVAC1.OVA in a ratio 1:9 for the DNA number of copies and co-precipitating on gold particles using a DLR of 2000 ng/mg of gold. Total amount of DNA/cartridge was $500 \pm 100 \text{ ng}$. Two cartridges (i.e. a total of $1000 \pm 200 \text{ ng}$) were delivered per immunisation (Figure 2.4). The unlinked DNA preparations were made by mixing pVAC1.NT with pVAC1 and pVAC1.OVA with pVAC1 in ratios of 1:9 and 9:1, respectively and co-precipitating those combinations on separate gold particles using a DLR of 4000 ng/mg of gold. Total amount of DNA/cartridge was $1000 \pm 200 \text{ ng}$ and 1 cartridge from each preparation was delivered to different

Linked DNA preparation



Unlinked DNA preparation

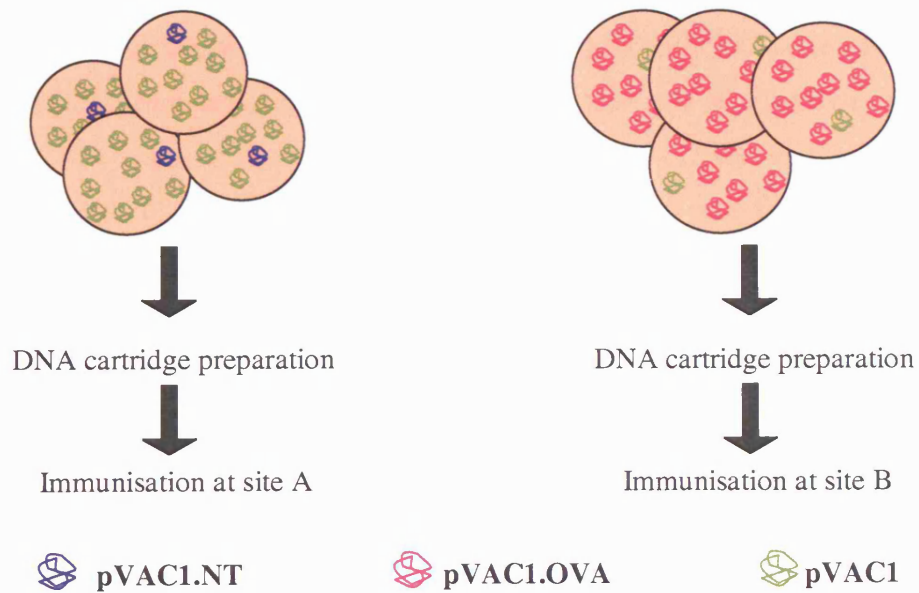


Figure 2.4. Schematic representation of linked and unlinked DNA cartridge preparations and immunisations.

immunisation sites (Figure 2.4). In this way, a total of 100 ng of pVAC1.NT and 900 ng of pVAC1.OVA were delivered in immunisations using cartridges made from either linked or unlinked DNA preparations. Linked DNA controls were made containing pVAC1.OVA or pVAC1.NT mixed with pVAC1 in ratios of 9:1 and 1:9, respectively.

2.5.3. DNA cartridges in dose response experiments

DNA cartridges for dose response experiments were made using linked DNA preparations. pVAC1.NT was titrated in log dilutions from 500-0.005 ng in either pVAC1.OVA or pVAC1. The dual plasmid combinations were co-precipitated on gold particles using a DLR of 2000 ng/mg of gold. Total amount of DNA/cartridge was 500 ± 100 ng. Three 50 μ l samples of DNA coated gold particles from each slurry batch were kept for quantitative PCR (QPCR) analysis.

2.5.3.1. QPCR analysis

QPCR analysis was performed on DNA eluted from gold particles. To remove the DNA, the gold particles were centrifuged at 14,000 rpm for 15 ± 5 sec using an Eppendorf 5415C centrifuge. The ethanol was removed and replaced by 45 μ l of DNase and RNase free water prior to incubation of each sample at 37°C and 5% CO₂ for 30 min. Elutions were repeated (using 50 μ l of RNase and DNase free water on the second occasion) and the two eluates pooled for analysis.

Eluted DNA was analysed for pVAC1.NT and pVAC1.OVA in separate QPCR assays. Probes and primers sequences (Perkin Elmer) shown in Table 2.2 were re-constituted to stock concentrations of 5 pM/ μ l and 22.5 pM/ μ l, respectively. The probes were flourigenically labelled with reporter, dT-FAM (excitation; 494 nm, emission; 517 nm) and quencher, TAMRA (excitation; 560 nm, emission; 583 nm). Optimised primer and probe concentrations for the pVAC1.NT assay were 90 nM forward primer (FP), 90 nM reverse primer (RP) and 250 nM probe. Optimised primer and probe concentrations used in the pVAC1.OVA assay were 270 nM FP, 900 nM RP and 200 nM probe, respectively. In the

pVAC1.NT assay, each reaction contained 2 μ l DNA eluate (1:10 or 1:100 dilution), 0.1 μ l each of primers, 1.56 μ l probe, 12.5 μ l master mix (Perkin Elmer) and 8.74 μ l of DNase and RNase free water. In the pVAC1.OVA assay, each reaction contained 2 μ l DNA eluate (1:10,000 dilution), 0.3 μ l FP, 1.0 μ l RP, 1.25 μ l probe, 12.5 μ l master mix (Perkin Elmer) and 7.95 μ l of DNase and RNase free water. Reactions were done as follows: 120 sec at 50°C, 600 sec at 95°C, 40 cycles (15 sec at 95°C, 60 sec at 60°C). Standard curves were generated from stock DNA used to prepare cartridges and ranged from 200,000-49 copies/ μ l. Dilutions of DNA eluates and standards were performed using DNase and RNase free water. Analysis was performed on an ABI 7700 sequence detector (PE Applied Biosystems).

2.6. *In vitro* transfection by particle-mediated DNA delivery

Transfection of RAW, J774, DAP-3/ICAM, EL4, MC57 and BMDC by PMDD was performed using either the suspension method or the plate method (Figure 2.5).

In the suspension method, a 20 μ l volume of cell suspension was transferred to the bottom of a 50 ml universal tube (Falcon). The cells were transfected by PMDD using the XR1 gene gun (PowderJect). Helium gas pressures ranging from 200-900 psi were used to discharge the DNA-coated gold from the cartridges into the cells. A range of distances (27-63 mm) of the gene gun from the cells was used. After transfection, the cells were washed with 1 ml of culture medium and transferred to 12 or 24 well plates for 24 h and at 37°C and 5% CO₂. In the experiments to investigate the effect of incubation time on transfection efficiency by PMDD using the suspension method, 9 individual samples were pooled directly after PMDD and plated out in 9 individual petri dishes (Sterilin) for incubation up to 24 or 106 h.

For the plate method, 20 μ l of cell suspension was evenly spread in the centre of a 6 well plate and transfected while holding the nozzle of the gene gun directly over the cells. A pressure range of 100-500 psi was used for the transfections. Once transfected, the cells

	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')
pVAC1.NT	-TGAGAGGGTCA GTTGCTCACA-	-TGGACGGCA TCGGTCACC-	-TTGTCTGCCC GCCTGTGTGT-
pVAC1.OVA	-GAGGAGGCTTGG AACCTATCAAC-	-AAGGACCCATCT TTCAGTCTGTTTA-	-CTGCAGATCAAGC CAGAGAGCTCATC-

Table 2.2. Probes and primers sequences used in the QPCR analysis of DNA eluates.

were washed with 1 ml of medium and transferred to 12 or 24 well plates for 24 h at 37°C and 5% CO₂. For both the suspension and plate PMDD methods, cell viability as estimated by Trypan blue (Sigma) exclusion was performed on cells after transfection.

2.7. Assessing transfection efficiency

Cells transfected by PMDD were assessed for transfection efficiency using the luciferase and *β-galactosidase* detection assays.

2.7.1. Luciferase assay

Cells were lysed by addition of 1 ml of cell culture lysis reagent (Promega) and stored at -80°C for 1 h. After thawing, 50 µl of cell lysate from each sample was assayed together with 50 µl of luciferase assay reagent (Promega) using either a Dynatech ML3000 microtiter plate luminometer or a Wallac Victor 1420 multi-label counter. An injection volume of 50 µl, sample delay time of 2 sec and data integration time of 20 sec was used. For the Dynatech luminometer, results were expressed as mean of duplicate or triplicate samples (relative light units (rlu)/ml). For the Wallac multi-label counter, results were expressed as mean of triplicate wells (counts per second (cps)/ml). Total protein concentration was calculated by Coomassie Plus protein assay reagent kit (Pierce). Ten µl of cell lysate was assayed together with 140 µl of water and 150 µl of Coomassie blue reagent in 96 well flat bottomed plates (Costar). The absorbance was measured at 595 nm on a Molecular Devices Spectra Max 340. Results were expressed as mean of triplicate readings (µg/ml). Luciferase activity was expressed as rlu or cps/µg of total protein (Figure 2.5) and reported as mean ± standard error mean of triplicate assays. All assays were performed using the Dynatech luminometer unless otherwise stated.

2.7.2. β -galactosidase assay

Transfected cells were tested for β -galactosidase activity 24 h after PMDD. The cells were washed once with 2 ml PBS, fixed with 1 ml of 10 % formaldehyde and incubated at RT for 10 min. The cells were washed 3 times in PBS (2 ml/well) before the addition of 1 ml/well staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride (all from Sigma) and 1 mg/ml X-galactosidase substrate (Promega)). The cells were incubated for 24 h at 37°C and 5% CO₂ and the total number of blue cells/well counted (mean of 10 random counts/well under x 20 magnification). Results were calculated as % of positive cells/viable cells plated out for each sample (Figure 2.5) and reported as mean \pm standard error mean of triplicate assays.

2.8. *In vitro* functional assays

In vitro functional assays were developed which used APC capable of presenting antigen to MHC matched effector cells.

2.8.1. Antigen presenting cells

In the MHC I functional assay, EL4, MC57 (normal and activated cultures) and BMDC from C57BL/6 mice were used as APC. To make activated MC57s, normal cultures were pre-treated with 20 ng/ml IFN- γ for 24 h prior to the *in vitro* assay. For the MHC II functional assay, DAP-3/ICAM cells and BMDC from BALB/c and D0.11.10 mice were used as APC. To make BMDC, cells were extracted from femurs, washed once in complete IMDM medium and cultured in complete IMDM containing 8 ng/ml of murine GM-CSF (Pharmingen) at 37°C and 5% CO₂ in sterile 90 mm Sterilin petri dishes. On day 3, the medium was replaced to remove the non-adherent cells. Six or 7 days later, the medium containing non-adherent cells (putatively BMDC) was transferred to new petri dishes and incubated for 1 h at 37°C and 5% CO₂. Contaminant macrophages adhered to the petri dish surface leaving the BMDC in the medium. The putative BMDC were subsequently

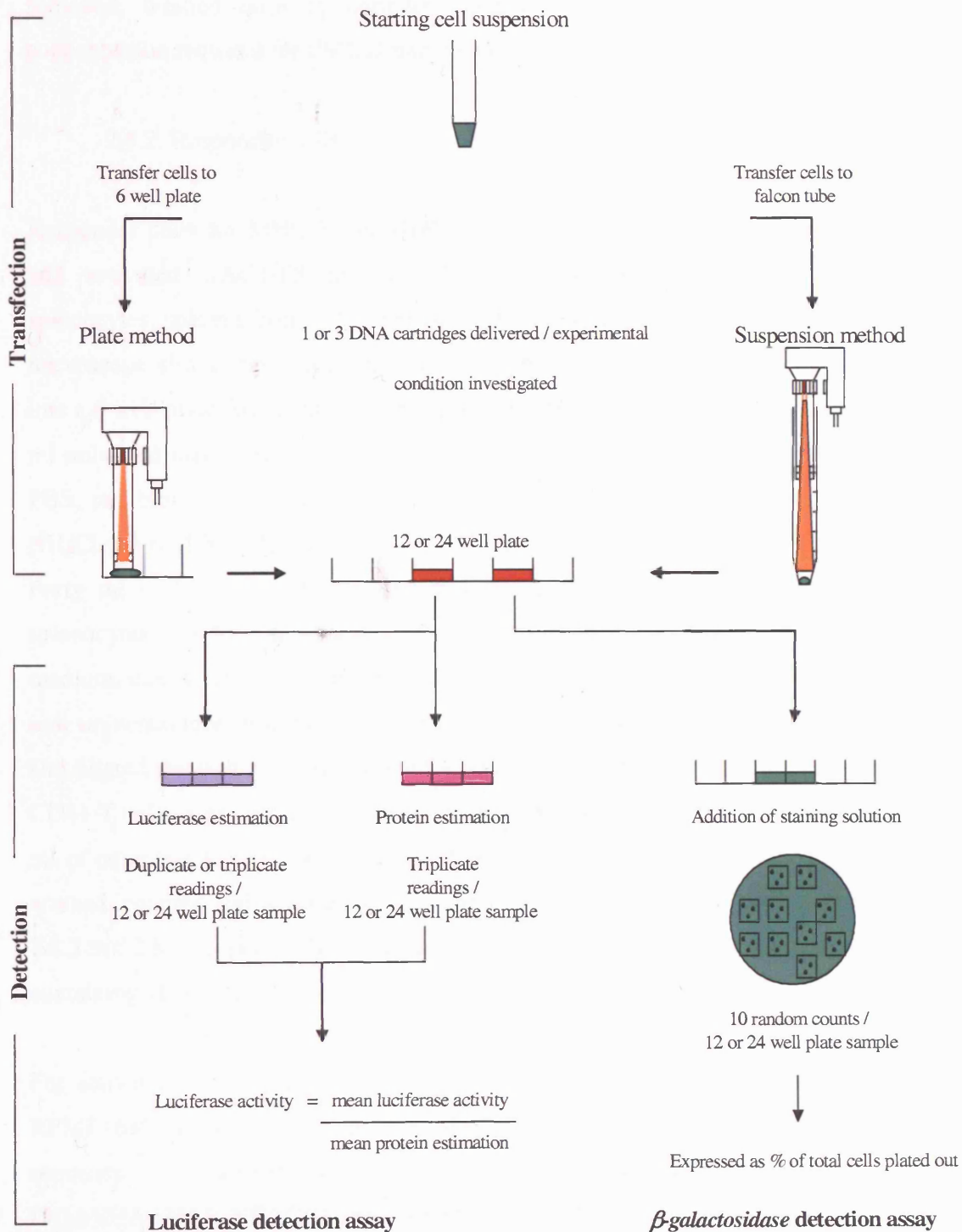


Figure 2.5. Schematic of both the plate and suspension PMDD transfection methods followed by luciferase and β -galactosidase reporter gene detection.

removed, washed once in complete IMDM medium, counted and adjusted to the concentration required for PMDD transfections.

2.8.2. Responder cells

Responder cells for MHC I and MHC II functional assays were naïve (freshly prepared) and activated RAG1/F5 and D0.11.10 splenocytes, respectively. To make naïve splenocytes, spleens from RAG1/F5 or D0.11.10 were teased between sterile ground glass microscope slides (super premium microscope slides, 1.0-1.2 mm thick/twin frost, BDH) into a 6 well plate well containing 5 ml sterile PBS. This suspension was transferred to a 50 ml universal tube (Falcon) and centrifuged at 1500 rpm for 5 min. After removal of the PBS, red blood cells were lysed by addition of 1 ml of filtered lysis buffer (155 mM NH_4Cl , 10 mM KHCO_3 and EDTA, all from Sigma) to the splenocyte pellet for 1-2 min. Forty ml of PBS was then added and the tube centrifuged again. For the RAG1/F5 splenocytes, this final splenocyte pellet was resuspended in 12 ml of complete RPMI 1640 medium, debris allowed to settle for ~1 min and the upper 10 ml of cells transferred to a new universal tube. For the D0.11.10 splenocytes, the pellet was resuspended in 10 ml PBS and filtered through a mouse CD4 subset column (R&D systems). The filtrate, enriched for CD4+ T cells, was centrifuged for 5 min at 1500 rpm and the cell pellet resuspended in 10 ml of complete RPMI 1640 medium. The RAG1/F5 and D0.11.10 splenocytes were then washed, counted and adjusted to the concentrations used in the *in vitro* assay (see sections 2.8.3 and 2.8.4, respectively) or adjusted to 1×10^7 cells/ml in freezing down medium (FCS containing 10% DMSO, Sigma) and stored at -20°C .

For activated splenocytes, 1×10^6 naïve splenocytes/well were stimulated with complete RPMI 1640 medium containing 500 nM peptide (RAG1/F5 splenocytes, A/NT/60/68₃₆₆₋₃₇₄, sequence ASNENMDAM; D0.11.10 splenocytes, OVA₃₂₃₋₃₃₉, sequence ISQAVHAAHAEINEAGR) and 20 ng/ml murine IL-2. Cells were incubated at 37°C and 5% CO_2 in 24 well plates. On day 3, cells were split 1:3 in complete RPMI 1640 medium containing 20 ng/ml IL-2 only. The cells were harvested on day 7, washed, counted and

adjusted to the concentrations used in the *in vitro* assay (see sections 2.8.3 or 2.8.4), or adjusted to 1×10^7 cells/ml in freezing down medium and stored at -20°C .

2.8.3. *In vitro* CD8+ T cell assay

On the day of assay, APC were transfected with plasmid encoding NT by PMDD (4×10^6 cells in a $20 \mu\text{l}$ volume using the plate method at 250 or 275 psi) and transferred in triplicate to pre-coated 96 well multiscreen-IP sterile ELISPOT plates (Millipore) at a cell density of 1×10^5 cells/well. RAG1/F5 splenocytes (freshly harvested or thawed from frozen stock) were added at a concentration of 1×10^5 cells/well to the APC. When RAG1.F5 splenocytes from frozen stock were used, the cells were thawed out at RT, washed 3 times in 10 ml of complete RPMI 1640, counted and adjusted to 1×10^5 cells/well. As positive controls, APC were pulsed with 100-200 nM of NT peptide (A/NT/60/68₃₆₆₋₃₇₄, sequence ASNENMDAM) by incubation with peptide for 1 hour at 37°C and 5% CO_2 , washed 3 times in PBS and plated. Negative controls were APC not pulsed with peptide and/or APC transfected with pVAC1 by PMDD. The assay was performed in complete RPMI 1640 medium alone or complete RPMI 1640 medium + IL-2 (200 ng/ml). When MC57 cells were used as APC, complete RPMI 1640 was replaced by MC57 growth medium (see section 2.2). The final volume/well was $200 \mu\text{l}$. After incubation at 37°C and 5% CO_2 overnight, cells were assayed for peptide specific CD8+ T cell activation by IFN- γ ELISPOT (section 2.9).

2.8.4. *In vitro* CD4+ T cell assay

On the day of assay, APC were transfected with pVAC1.OVAs by PMDD (4×10^6 cells in a $20 \mu\text{l}$ volume using the plate method at 250 or 275 psi) and transferred in triplicate to pre-coated 96 well multiscreen-IP sterile ELISPOT plates (for IL-2 ELISPOT assay) and/or to 96 well Costar flat bottomed plates (for IL-2 ELISA and T cell proliferation assays) at a cell density of 2×10^5 cells/well. D0.11.10 splenocytes (freshly harvested or thawed from frozen stock) were added at a concentration of 2×10^5 cells/well to the APC. DAP-3/ICAM cells pulsed with OVA peptide (OVA₃₂₃₋₃₃₉, sequence ISQAVHAAHAEINEAGR) or

protein (1000-10 nM) were positive controls. Negative controls were APC not pulsed with peptide and/or APC transfected with pVAC1 by PMDD. Final volume/well was 200 μ l. After incubation at 37°C and 5% CO₂ overnight, cells were assayed for peptide specific CD4+ T cell activation by IL-2 ELISPOT (section 2.9) and/or IL-2 ELISA (section 2.10) and thymidine incorporation assays (section 2.11).

2.9. Cytokine detection-ELISPOT

96 well multiscreen-IP sterile ELISPOT plates were pre-coated with purified rat IgG_{2a} anti mouse IL-2 (clone JES6-1A12) or rat IgG₁ anti mouse IFN- γ (clone XMG1.2) at a concentration of 15 μ g/ml (50 μ l/well) by incubation at 4°C overnight. The plates were blocked with complete RPMI 1640 medium for 1 h at 37°C and 5% CO₂ before sample addition. After incubation of samples (for both *in vitro* and *in vivo* experiments) at 37°C and 5% CO₂ overnight, plates were washed with PBS, distilled water (100 μ l/well for 5 min at RT) and a further 3 washes with PBS. Detection antibodies were biotin conjugated rat IgG_{2b} anti mouse IL-2 (clone JES6-5H4) or rat IgG₁ anti mouse IFN- γ (clone R4-6A2). They were added for 2 h at RT at a concentration of 1 μ g/ml (50 μ l/well) while shaking. Streptavidin alkaline phosphatase conjugate (Caltag) at a 1:1,000 dilution was added and incubated for 2 h at RT (50 μ l/well) while shaking. All antibodies were purchased from Pharmingen unless otherwise stated. Three washes with PBS were performed between each step. Fifty μ l/well of alkaline phosphatase conjugated substrate (BIO-RAD) was used to develop the assay and the assay stopped by the addition of water once spots appeared (usually between 10 and 30 min after substrate addition). After drying at RT overnight, cytokine producing cells were enumerated by an image analysis system developed by GSK. For *in vitro* experiments, number and/or area of cytokine spots (normalised to spots/million cells) were calculated and results reported as mean \pm standard deviation of triplicate wells. For *in vivo* experiments, number of cytokine spots (normalised to spots/million cells) were calculated and results reported as mean \pm standard error mean of triplicate samples.

2.10. Cytokine detection-ELISA

Purified rat IgG_{2a} anti mouse IL-2 (clone JES6-1A12) was coated on Nunc maxisorb 96 well plates at a concentration of 2 µg/ml in PBS (50 µl/well) and incubated at RT overnight. Non specific binding was blocked by addition of 50 µl/well of PBS with 1 % BSA (Sigma) and incubation for 1 h at RT. Fifty µl of culture medium supernatant (1:5 dilution) collected after the incubation of APC and T cells (see section 2.8.4) and recombinant murine IL-2 (Pharmingen) were added and incubated for 2 h at RT while shaking. Detection antibody was biotin conjugated rat IgG_{2b} anti mouse IL-2 (clone JES6-5H4) and was added at a concentration of 1 µg/ml (50 µl/well) while shaking. After 2 h at RT, streptavidin-HRP conjugate (Caltag) at a 1:2,000 dilution was added and left for 30 min in the dark at RT (50 µl). Three washes with PBS containing 1% BSA were performed between each step. Fifty µl/well of tetramethyl benzidine substrate (Sigma) was used for colour development, the reaction stopped with 1 N H₂SO₄, and plates read at 450 nm on a Molecular Devices Spectra Max 340. Results were reported as mean ± standard deviation of triplicate wells.

2.11. T cell proliferation

Fifty µl of complete RPMI 1640 medium was added back to the 96 well plates to replace that taken for IL-2 ELISA analysis (see section 2.10). The cells were pulsed with 20 µl of low activity ³H thymidine (33 KBq/well) (Amersham Life Sciences). After incubation overnight at 37°C and 5% CO₂, the cells were harvested and thymidine incorporation measured using a liquid scintillation counter (Wallac 1205 Betaplate). Results were reported as mean ± standard deviation of triplicate wells.

2.12. Western blot analysis

MC57 cells were transfected with plasmid encoding NT by PMDD (4 x 10⁶ cells in a 20 µl volume using the plate method at 275 psi), adjusted to 1 x 10⁶ cells/ml in MC57 growth medium and incubated overnight at 37°C and 5% CO₂ in 24 well plates (1 ml cells/well).

For each sample, cells were removed from wells, transferred to Eppendorf tubes, washed once in 500 μ l of PBS and resuspended in 100 μ l of PBS for analysis. Samples were run on a NuPAGE Novex gel (Invitrogen). Sixteen μ l of sample were incubated with 5 μ l of 4 x buffer and 1.5 μ l of 10 x buffer (both from NuPAGE lysis kit, Invitrogen) for 10 min at 80°C and 20 μ l of sample loaded on the gel. For positive control, 1 μ l of PR-GST fusion protein (1 mg/ml) was incubated with 9 μ l of water, 5 μ l of 4 x buffer and 1.5 μ l of 10 x buffer and 5 μ l loaded on the gel. For internal protein marker, 5 μ l of SeeBlue protein (4-250 kDa ladder, Invitrogen) was added to 2 or 3 wells. The gel was run for 35 min at 200 volts in running buffer and on completion, transferred to an Immobilon P membrane (Millipore) in transfer buffer for 1 h at 30 volts. Running buffer and transfer buffer were made by diluting 10 x NuPAGE MES running buffer and 10 x NuPage transfer buffer (both from Invitrogen) 1:10 in deionised water, respectively. Next, 10 ml of block buffer (PBS with 0.1% Tween and 100 mg/ml condensed milk, both from BDH) was added to the blot for 1 h at RT. The blot was incubated for 2 h with primary antibody (rabbit serum anti NT-GST fusion, 1:500 dilution) and 1 h with secondary antibody (swine anti rabbit immunoglobulin-HRP (Dako), 1:20,000 dilution). Both incubations were performed in 10 ml of block buffer at RT. Four washes in PBS containing 0.1% Tween were done between each step. Then, 6 ml of ECL detection substrate (BioWest or Amersham) was added to the blot for 5 min at RT while gently shaking. Finally, the blot was placed between plastic sheets and exposed on a EpiChemii darkroom (Ultra-Violet Products) and/or a Fugii X-Ray developer using Hyperfilm™ ECL™ film (Amersham).

2.13. Immunisations

Immunisations were kindly performed by Dr L Thomsen, Dr F Cook, Dr G Bembridge or Dr K Barber, GSK, U.K. Recipient mice (3 per group), 4 to 10 weeks of age, were shaved on their abdomen and given 2 cartridges, one cartridge on either side of the ventral mid-line. PMDD was performed using the Powderject XR1 device. Helium pressure of 500 psi was used to discharge the DNA (typically 500 ng/cartridge) from the cartridges to the mice. For immunisations involving unlinked DNA preparations, (refer to section 2.5.2 and Figure

2.4), one cartridge containing one plasmid was delivered to one side of their abdomen and the second cartridge containing the second plasmid was delivered to the other side.

2.13.1. Cytokine producing cells

On the day of analysis, spleens were removed and single cell suspension of splenocytes prepared. The method used was that described for naïve RAG1/F5 splenocytes (section 2.8.2). Cytokine producing cells were analysed by ELISPOT as described in section 2.9. For each assay, 2 or 4 x 10⁵ splenocytes (100 µl/well) were assayed in triplicate. The following combinations were added to give a final volume of 200 µl/well: complete RPMI 1640 medium + 50 ng/ml IL-2 or complete RPMI 1640 medium + 50 ng/ml IL-2 + peptide. NT peptide, ASNENMDAM, or OVA peptide, SIINFELK were added at 100 nM. NT peptide, SVQRNLPFDKPTIMAAFTGNTEG, OVA peptide, TEWTSSNVMEERKIKV or HPV 6 E1 protein were added at 20 µM and HIV polymerase protein was added at a concentration of 10 µg/ml. The concentrations of NT peptides, OVA peptides and HPV 6 E1 and HIV protein used were previously determined by Dr K Barber, Dr L Thomsen and Dr F Cook, respectively. Results (mean ± standard error mean) for each group of mice were calculated as the means of individual means of triplicate results for each individual mouse.

2.13.2. Intracellular cytokine staining

Aliquots of sample prepared for ELISPOT analysis (see section 2.13.1) were taken for ICS analysis. Pooled splenocytes (2 x 10⁶) were incubated for 6 h at 37°C and 5% CO₂ in flow cytometry tubes (Beckman Coulter) containing 1 ml of complete RPMI 1640 medium with 100 nM NT peptide, sequence ASNENMDAM and 50 ng/ml IL-2. After 2 h, Brefeldin A (Sigma) was added to a final concentration of 10 µg/ml. After incubation for the remaining 4 h, they were centrifuged for 5 min at 1500 rpm, washed with 3 ml of FACS buffer (PBS containing 1% FCS) and surface stained with rat IgG2a anti mouse CD62L-FITC (Pharmingen, clone MEL-14, 0.125 µg/sample) and rat IgG2a anti mouse CD8-Cy-Chrome (Pharmingen, clone 53-6.7, 0.5 µg/sample). Staining was done in FACS buffer (100 µl/sample) for 20 min at 4°C. Cells were washed twice and kept overnight at 4°C in

fixative solution (Intra prep. kit, Immunotech, 100 μ l/sample). Next, they were washed twice and stained for rat IgG1 anti mouse IFN- γ -PE (Pharmingen, clone XMG1.2, 0.1 μ g/sample) for 15 min at RT in permeabilisation solution (Intra prep. kit, 100 μ l/sample). Finally, they were washed twice in FACS buffer and resuspended in an 800 μ l volume for analysis. Flow cytometry analysis was performed on a Beckman Coulter EPICS XL using system II acquisition software.

2.14. Statistical analysis

For *in vitro* analyses, Dunnett's test or Student's T-test was performed in experiments that were repeated at least three times. For *in vivo* analyses, Student's T-test was performed in all experiments where applicable.

The Dunnett's test is a multiple comparison procedure for comparing each mean against a standard control group mean. The Student's T-test is used to test hypotheses between population means. For the *in vivo* experiments, 3 mice/group were routinely used. Because of this limited sample size, the degrees of freedom associated with these groups are small thus making it difficult to detect significance between group means.

The *in vivo* and *in vitro* immunising dose and promoter dose responses were compared using correlation analysis. Mean values was used for correlation and linear trend-line regression coefficient (R^2) was fitted to the data.

Chapter 3

Optimisation of PMDD *in vitro*

3.1. Introduction

The ability of the adaptive immune response to develop immunological memory forms the basis for the successful application of vaccination in human health-care. In the past, many vaccine strategies have been developed to eradicate infectious diseases with varying degrees of success. Recently, taking advantage of developments in recombinant DNA technology and molecular biology, research in the field of vaccination has focused on the development of DNA vaccines in various forms. Before such vaccines can be successfully utilised, prophylactically or therapeutically in man, many important issues need to be addressed. These issues centre not only upon the biological potency of DNA vaccines themselves and their ability to evoke a complete and balanced immune response, but also on the efficacy of the technology used to deliver the DNA.

This is especially pertinent to PMDD where compressed helium gas is used to accelerate microscopic gold beads coated with DNA into the target tissue. Optimal delivery is dependent on a number of variables, some of which may be manipulated or improved to enhance the efficiency of delivery. These variables may be broadly categorised as ballistic (i.e. associated with the PMDD process) or non-ballistic (i.e. independent of the PMDD process). Given that responses to DNA vaccines in man are at present unsatisfactory, optimising these parameters will help play an important role in determining the success of this type of vaccine strategy.

Since the emergence of PMDD as a vaccination tool with implications in the clinic, the application of this technique for the transfection of mammalian cells has also gained interest. It was first pioneered as a means of introducing genetic material into plant cells (Klein *et al.*, 1987) and now the technique assumes an important role as a transfection tool in this field. Due to improvements in device technology and more importantly, its direct applicability to the *in vivo* transfection process, PMDD to mammalian cells *ex vivo* and *in vitro* is becoming the subject of an increased amount of research. In terms of efficiency, it compares favourably with lipofection and calcium phosphate transfection (Thompson *et al.*, 1993; Yang *et al.*, 1990; Heiser, 1994; Guo *et al.*, 1996). It is not as efficient as viral

transfection techniques but it is easier and quicker to use and does not suffer from the same complications associated with gene delivery. Unlike most other transfection methods, PMDD is a mechanical way of introducing genes across the plasma membrane and therefore is not affected to the same extent as other methods by the characteristics of the targeted cell.

However, there are still inconsistencies associated with the technique which prevent its more widespread use. Significantly, PMDD to mammalian cells *in vitro* has employed different delivery devices and reports have incompletely defined the extent of experimental parameter optimisation (Fitzpatrick-McElligott, 1992; Zelenin *et al.*, 1989). Furthermore, transfected cell lines show different expression rates for the introduced gene (Gainer *et al.*, 2000) highlighting the requirement for the assessment of each individual cell line for each gene introduced. In this chapter, the effect of various ballistic and non-ballistic variables on PMDD transfection efficacy has been assessed. Different methods of PMDD to mammalian cells of varying lineage *in vitro* were investigated and compared using the β -galactosidase and firefly luciferase reporter genes.

3.2. Objective

- To develop and optimise a PMDD method for transfection of mammalian cells *in vitro* using the PowderJect XR1 delivery device.
- To investigate PMDD transfection efficiency in a range of cell lines.
- To identify suitable cell lines on the basis of transfection efficiency for subsequent use in *in vitro/in vivo* correlation studies.

3.3. Results

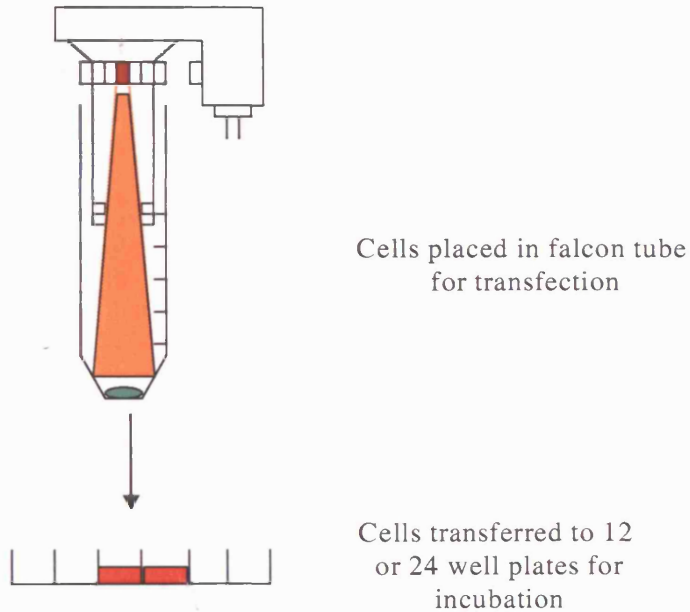
3.3.1. *In vitro* transfection by PMDD using the suspension method

Optimisation of *in vitro* transfection by PMDD using the suspension method (described in Figure 3.1) was performed using mouse macrophage cell lines, RAW and J774 and the transformed fibroblast cell line DAP-3/ICAM. Process variables investigated were cell suspension volume and concentration of cells transfected, device positioning, PMDD pressure and incubation time after PMDD. The effect of cell suspension volume and concentration of cells transfected on PMDD efficiency is shown in Figure 3.2, panel A. The data indicates that for both J774 and RAW cell lines, a higher cell concentration (1×10^7 cells) was optimal for transfection efficacy while altering the cell suspension volume had less influence on the level of transfection. Neither parameter affected cell viability. RAWs transfected much more efficiently than J774 cells and were less susceptible to cell death following the transfection process (~7.0 % for RAWs compared with ~13.3 % for J774s).

The effect of device positioning on transfection efficacy is represented in Figure 3.2, panel B and shows that optimal transfection was achieved when the PMDD device was positioned closest to the cell suspension (i.e. 27 mm from the RAW suspension and 27-36 mm from the J774 cell suspension). Cell death was at its greatest at the 27 mm distance (~28.0 % for RAW and ~31.3 % for J774). As expected, there was an overall decrease in transfection efficiency and cell death as the device was moved away from the cells.

For PMDD pressure, transfection efficacy increased with increasing PMDD pressure to an optimal delivery pressure of 800 psi in one assay and 900 psi in the other two (Figure 3.3, panel A). The accompanying cell death data (Figure 3.3, panel B) was generally low (<10 %) except at 900 psi where the mean of three assays was ~13 %. The final variable investigated was that of incubation time after PMDD. Two separate time-course experiments were performed using DAP-3/ICAM cells only (0-24 h) and DAP-3/ICAM and RAW cells (8-106 h). In the 24 h time-course experiment, luciferase expression

PMDD using the suspension method



PMDD using the plate method

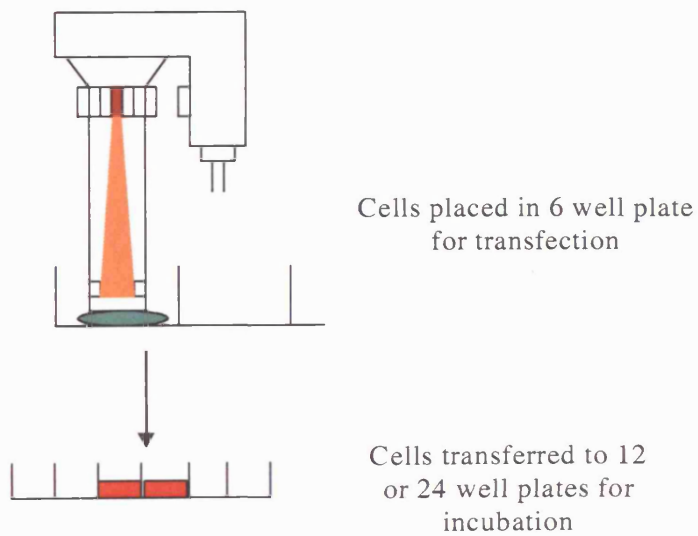


Figure 3.1. Schematic representation of suspension and plate PMDD transfection methods.

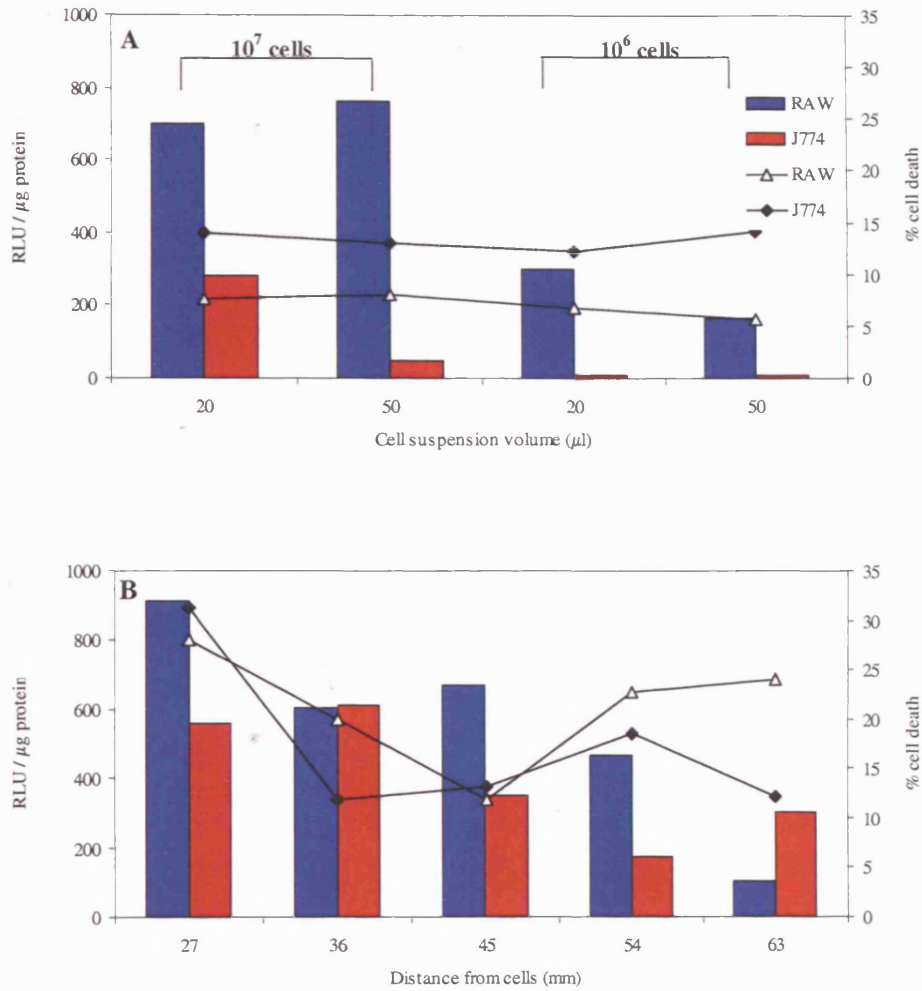


Figure 3.2. The effect of cell suspension volume and cell concentration (Panel A) and device positioning (Panel B) on PMDD efficiency using the suspension method. PMDD of pGL3-CMV-luciferase was carried out at 500 psi using RAW and J774 cells. In A, the delivery device was positioned 63 mm from the cell suspension. In B, a cell suspension volume of 20 μl and cell concentration of 4×10^6 cells was used. For both A and B, luciferase activity was measured 3.5 h after PMDD with results expressed as rlu/ μg protein. Data are from one experiment and represent mean of duplicate analyses. Bars represent data for luciferase activity. Lines and symbols represent cell viability data.

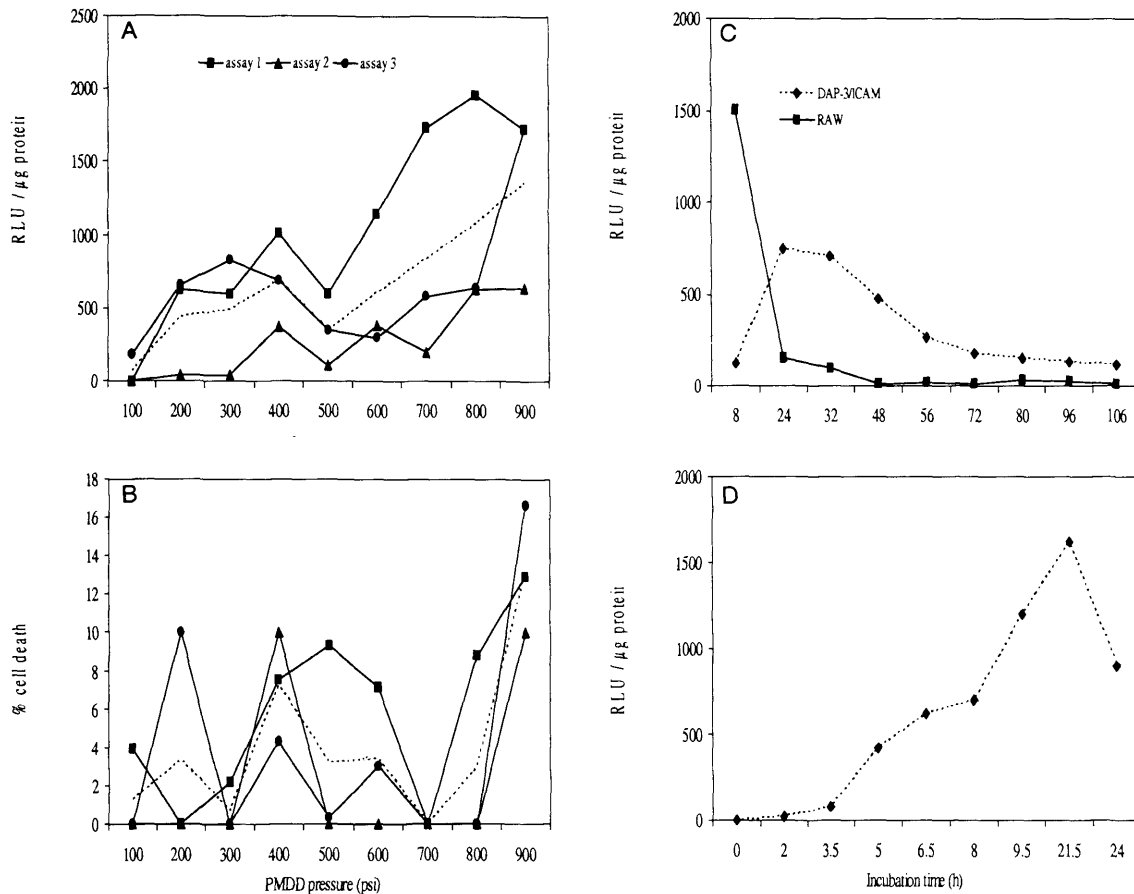


Figure 3.3. The effect of PMDD pressure on transfection efficiency (A) and cell viability following PMDD (B) using the suspension method. PMDD of pGL3-CMV-luciferase was carried out using DAP-3/ICAM cells (4×10^6 cells in $20 \mu\text{l}$) with the delivery device positioned 27 mm from the cells. Luciferase activity was measured 24 h after PMDD with results expressed as rlu/ μg protein. Data are from three separate experiments and represent mean of triplicate analyses of one transfection suspension. Broken line joins the means of all experiments. Panels C and D: effect of incubation time on PMDD transfection efficiency using the suspension method. PMDD of pGL3-CMV-luciferase was carried out using DAP-3/ICAM and RAW cells (C) or DAP-3/ICAM cells only (D) (4×10^6 cells in $20 \mu\text{l}$) at 500 psi with the delivery device positioned 27 mm from the cells. Luciferase activity was expressed as rlu/ μg protein. For both C and D, data are from one experiment and represent mean of triplicate analyses of one transfection suspension.

increased continually to a maximum at 21.5 h (Figure 3.3, panel D) and then decreased. This is in accordance with the longer time course experiment (Figure 3.3, panel C) which shows an increase in luciferase expression up to 24 h followed by a gradual decrease to a background level of expression after ~72-80 h. Collectively, the data infers an optimal incubation time of between 20 and 24 h for DAP-3/ICAM cells. In contrast, RAWs cells appear to reach a peak level of expression much earlier (at least by 8 h) which decreases to a background level of expression after 48 h.

3.3.2. Comparison of suspension and plate methods

Preliminary experiments with the suspension method suggested that transfection was best with a high cell concentration (i.e. $\geq 4 \times 10^6$ cells) in a small volume (i.e. 20 μ l) with the device positioned close to the cells (i.e. ≤ 27 mm). Under these conditions, optimal transfection efficacy was achieved at pressures of ≥ 800 psi (Figure 3.3, panel A). For the plate method (described in Figure 3.1), optimal luciferase expression was achieved using PMDD pressures of 250-350 psi (Figure 3.4). Both data sets appear to show similar levels of inter-assay variability, especially around the optimal PMDD pressures (i.e. 700-900 psi for the suspension method and 200-350 psi for the plate method). Highest average reading for luciferase expression was ~1365 and ~1400 rlu/ μ g protein for the suspension method (900 psi) and plate method (250 psi), respectively.

To make a more accurate comparison of both transfection methods, DAP-3/ICAM cells were transfected with β -galactosidase and luciferase plasmids by both methods using the same conditions and reporter gene expression (Figure 3.5, panels A and B) and cell death following transfection (Figure 3.5, panels C and D) were assessed. Optimal β -galactosidase and luciferase expression was obtained at PMDD pressures of 700 and 900 psi for the suspension method and at 200 and 300 psi for the plate method, respectively. In both assays, the maximum level of reporter gene expression using the plate method exceeded that of the suspension method. Cell viability using the suspension method was high in both the β -galactosidase and luciferase assays (Figure 3.5, panels C and D) over the PMDD

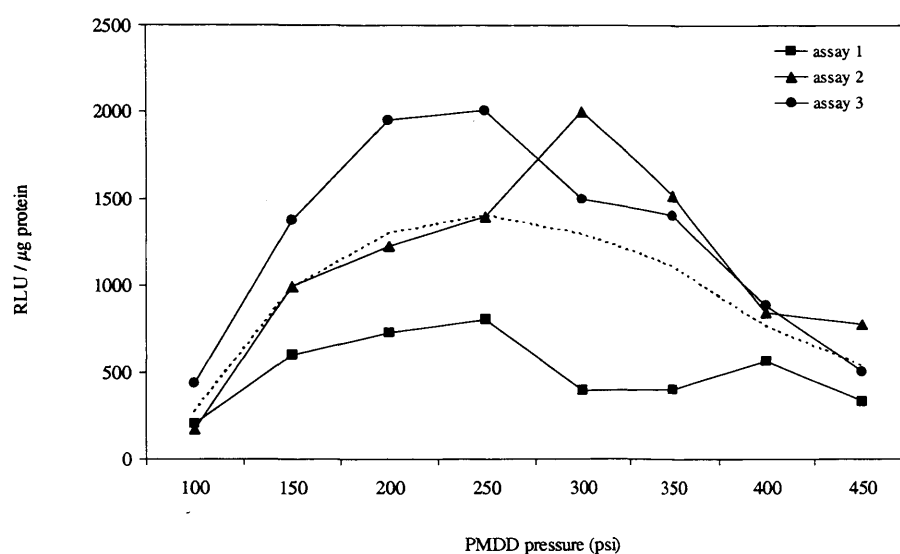


Figure 3.4. The effect of PMDD pressure on transfection efficiency using the plate method. PMDD of pGL3-CMV-luciferase was carried out using DAP-3/ICAM cells (4×10^6 cells in $20 \mu\text{l}$) with the delivery device positioned against the base of the tissue culture well directly over the cells. Luciferase activity was measured 24 h after PMDD with results expressed as $\text{rlu}/\mu\text{g}$ protein. Data are from three separate experiments and represent mean of triplicate analyses of one transfection suspension. Broken line joins the means of all experiments.

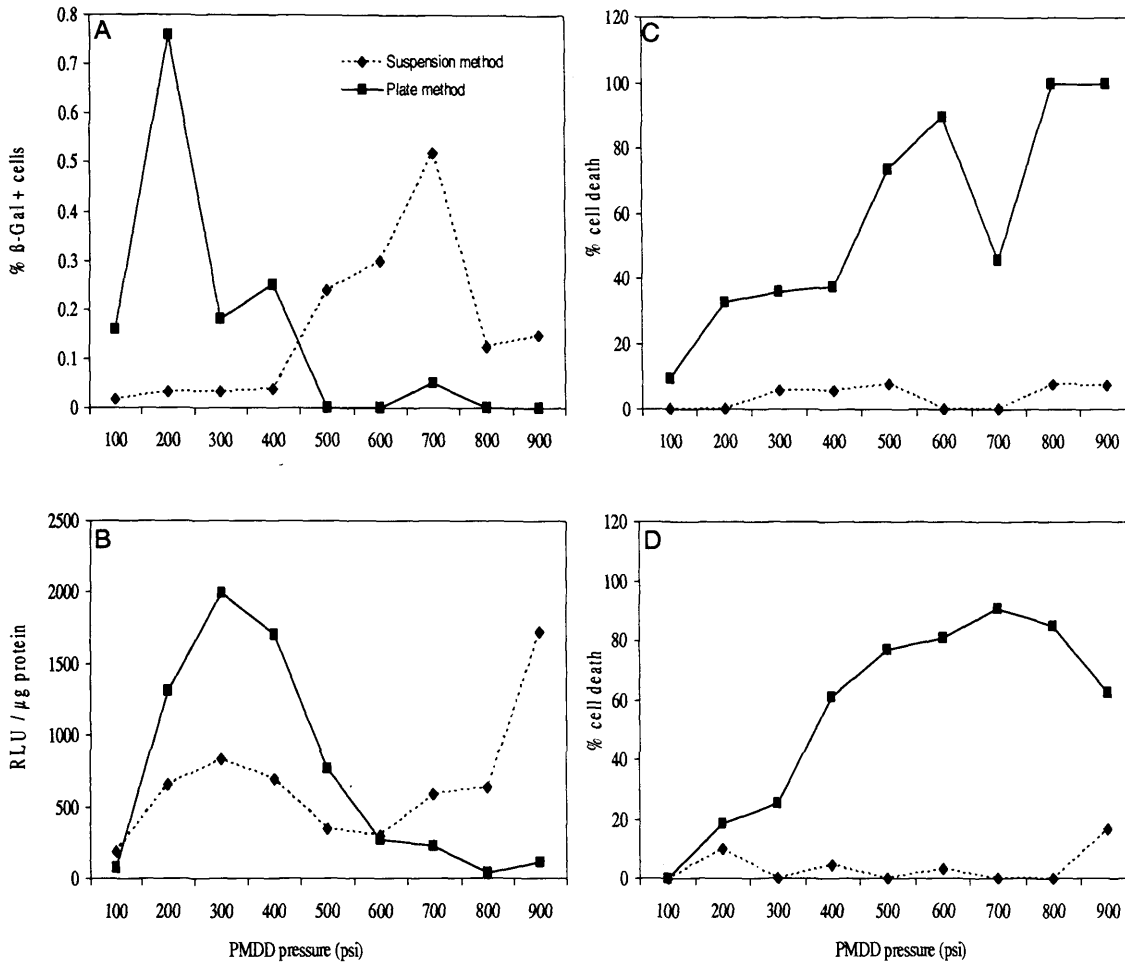


Figure 3.5. Comparison of PMDD suspension and plate methods. PMDD of pCIneo-CMV- β -galactosidase and pGL3-CMV-luciferase was performed using DAP-3/ICAM cells (4×10^6 cells in $20 \mu\text{l}$) with the delivery device positioned 27 mm from the cell suspension for the suspension method or against the base of the tissue culture well directly over the cells for the plate method. The effect of PMDD pressure on β -galactosidase and luciferase transfection efficiency is shown in panels A and B, respectively. The effect of PMDD pressure on cell viability following PMDD of β -galactosidase and luciferase plasmids is shown in panels C and D, respectively. Activity was measured 24 h after PMDD with β -galactosidase results expressed as % of positive cells total viable cells plated out and luciferase results expressed as rlu/ μg protein. Data are from one experiment and represent mean of triplicate analyses of one transfection suspension.

pressure range investigated. However, when the plate method was employed for transfection, increasing the PMDD pressure above 400 psi in both assays resulted in near total cell death.

3.3.3. Evaluation of transfection ability of a range of cell lines

Cell lines EL4, MC57 and BMDC purified from BALB/c mice were assessed for transfection efficiency using the plate method and the β -galactosidase reporter plasmid (Figure 3.6). Results demonstrate that both EL4 and MC57 cells are transfectable while the BMDC prove refractory to transfection. The EL4 cells transfected at frequencies of ~1.4 and ~0.1 and MC57 cells at ~3.2 and ~8.3 % in assay one (using cartridge preparation A) and two (using cartridge preparation B), respectively. Representative β -galactosidase transfections of EL4 and MC57 cells from assay two is shown in Figure 3.6, panels B-E. The data shows that there is considerable inter and intra-assay variation within these assays, a feature previously reported (Yang *et al.*, 1990). DAP-3/ICAM and FS-DC cells were assessed for transfection efficiency in separate studies transfecting at ~0.8 % (Figure 3.5, panel A) and 0 % (data not shown), respectively.

3.3.4. Further optimisation of transfection of the MC57 cell line

Transfection of the MC57 cell line was investigated further and optimal transfection conditions were more completely defined. Initially, incubation time after PMDD was optimised, followed by cell resting time after PMDD (i.e. incubation time between transfection and resuspension of cells in medium after transfection), then PMDD pressure and finally concentration of cells transfected. Each condition was investigated using luciferase and/or β -galactosidase reporter plasmids. Luciferase detection for these assays was performed using a Wallac Victor 1420 Multi-label counter and the cell death data represented is that associated with the β -galactosidase transfection assays. For the effect of incubation time after PMDD on transfection efficacy, reporter gene expression was assessed from 12-28 h post PMDD (at 4 hourly intervals). In general, a relatively constant

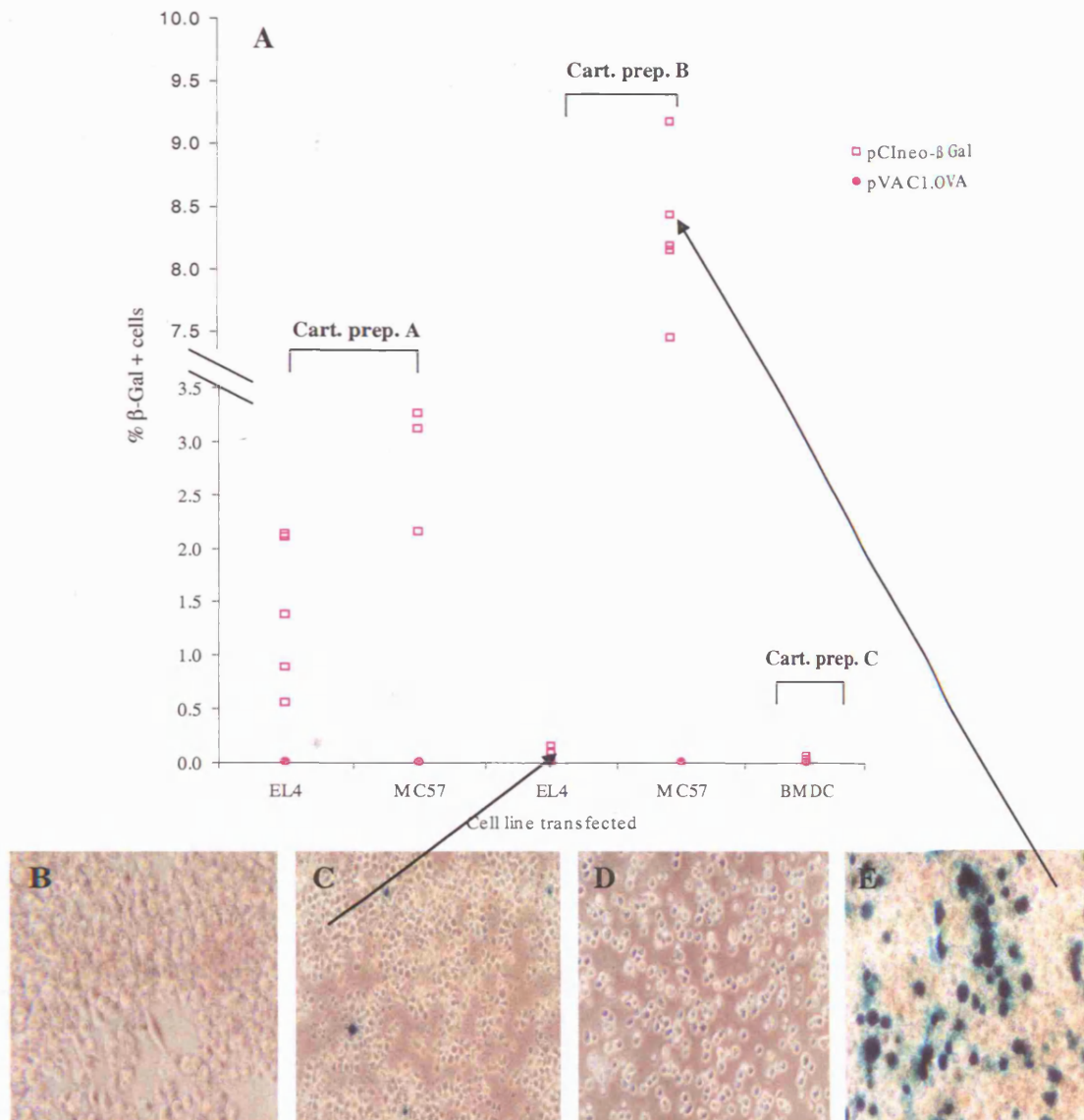


Figure 3.6. Transfection of EL4 (n=5), MC57 (n=5) and BMDC (n=4) with pCIneo-CMV-β-galactosidase using the plate method (panel A). PMDD was performed at 250 psi using 4×10^6 cells in 20 μ l with the delivery device positioned against the base of the tissue culture well directly over the cells. β-galactosidase activity was measured 24 h after PMDD with results expressed as % of positive cells/total viable cells plated out. Panels B-E are representative photographs after β-galactosidase staining of pVAC1.OVAs transfected EL4 cells, pCIneo-CMV-β-galactosidase transfected EL4 cells, pVAC1.OVAs transfected MC57 cells and pCIneo-CMV-β-galactosidase MC57 cells, respectively. Photographs were taken using a Nikon CF PL with Kodak NC film (magnification: X 250).

level of reporter gene expression was obtained over the time-course investigated with average readings for luciferase ($\sim 21,440$ cps/ μ g protein) and β -galactosidase expression (~ 4.0 %) peaking at the 20 h time-point (Figure 3.7, panels A and B, respectively). For cell resting time after PMDD (0-15 min) results for luciferase expression showed an average maximum level of expression (14,210 cps/ μ g protein) using a resting time of 6 min (Figure 3.8, panel A). In contrast, the β -galactosidase transfections recorded the highest average reading (~ 1.8 %) at a resting time of 9 min (Figure 3.8, panel B). The cell death data indicated that cell viability is not affected by cell resting time with average values remaining constant over the cell resting time range investigated (Figure 3.8, panel C).

The contribution of PMDD pressure on transfection efficacy was also investigated. Cells were transfected using a PMDD pressure range of 100-450 psi. Optimal luciferase expression was obtained at 300 psi, with all assays exhibiting a similar trend over the PMDD pressure range investigated (Figure 3.9, panel A). For β -galactosidase expression optimal expression was observed at 350 psi in two assays and 200 psi in the third (Figure 3.9, panel B). The highest average values for luciferase and β -galactosidase expression were $\sim 17,710$ cps/ μ g protein and ~ 1.7 % at 300 and 200 psi, respectively. Viability of cells decreased with increasing PMDD pressure (Figure 3.9, panel C) with a difference of 25.1 % in the average cell death between the highest and lowest PMDD pressures used (~ 79.7 % at 450 psi and ~ 54.6 % at 100 psi).

The final variable optimised was cell concentration. Three concentrations of cells (2, 4 and 8×10^6 cells in a 20 μ l volume) were transfected using the luciferase reporter plasmid. Although optimal luciferase expression was achieved at a cell concentration of 4×10^6 cells/20 μ l in two of the three assays performed, the highest average value for luciferase expression ($\sim 28,040$ cps/ μ g protein) was obtained at 2×10^6 cells/20 μ l (Figure 3.10). Optimal conditions for PMDD to MC57 cells were identified as follows: incubation time after PMDD of 20 h, cell resting time of 6–9 min, PMDD pressure of 200-300 psi and cell concentration of $2-4 \times 10^6$ cells/20 μ l.

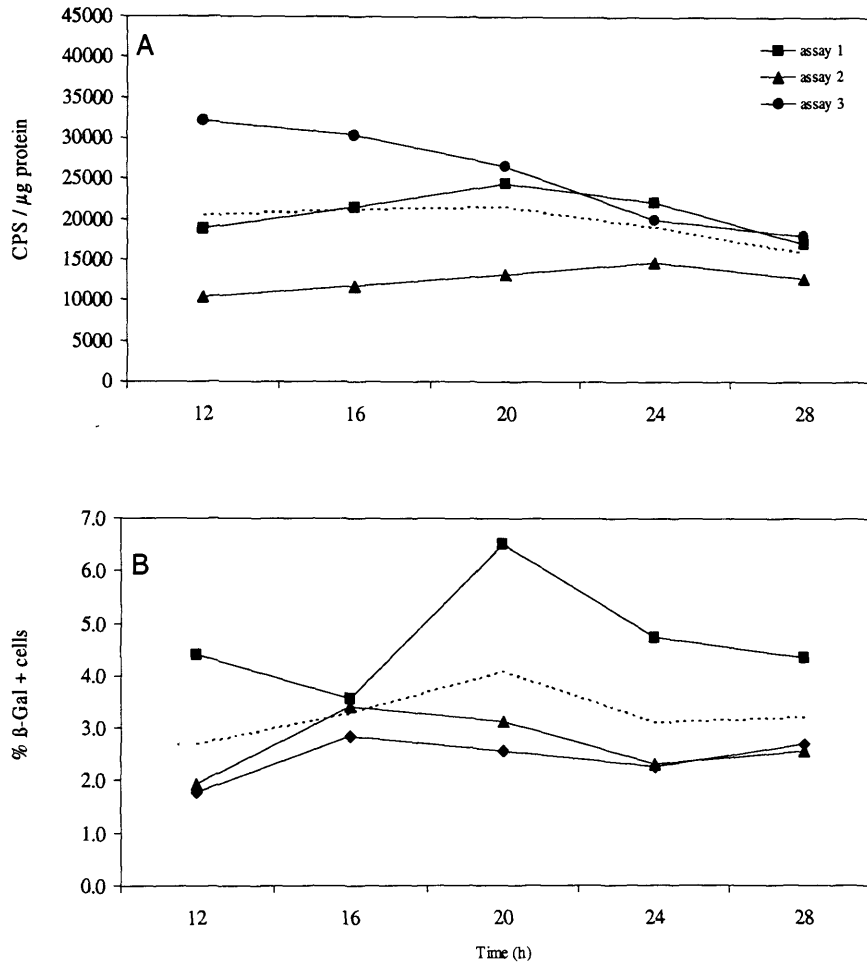


Figure 3.7. The effect of incubation time after PMDD on luciferase and β -galactosidase transfection efficiency in MC57 cells is shown in panels A and B, respectively. PMDD of pGL3-CMV-luciferase and pCIneo-CMV- β -galactosidase was performed at 250 psi using the plate method (8×10^6 cells in $20 \mu\text{l}$) with the delivery device positioned against the base of the tissue culture well directly over the cells. Cell resting time of 9 min was used. Activity was measured 24 hrs after PMDD with β -galactosidase results expressed as % of positive cells/total viable cells plated out and luciferase results expressed as cps/ μg protein. For each experiment, data represent mean of triplicate analyses of three transfection suspensions. Broken line joins the means of all experiments on each graph.

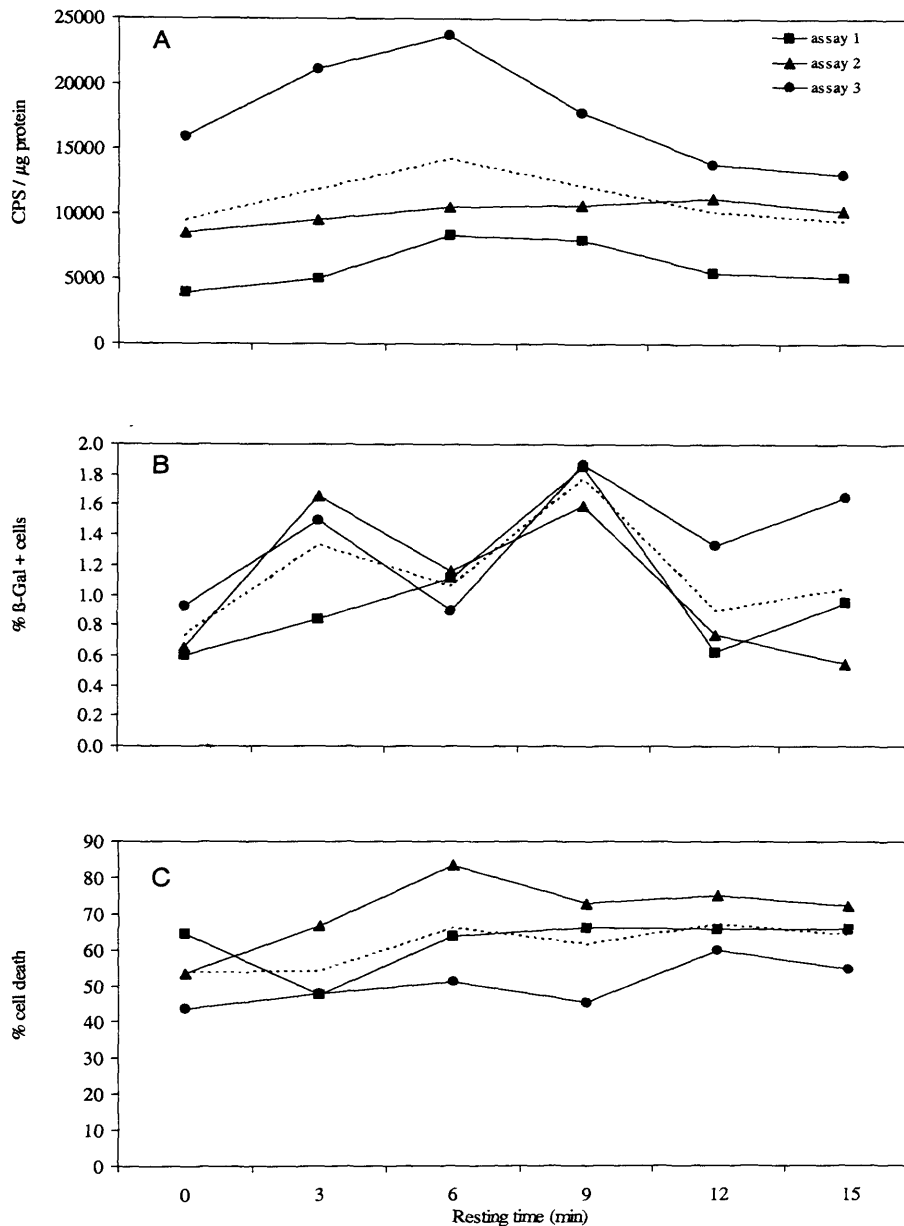


Figure 3.8. The effect of cell resting time on luciferase and β -galactosidase transfection efficiency in MC57 cells is shown in panels A and B, respectively. The effect of cell resting time on MC57 cell viability following PMDD of β -galactosidase is shown in panel C. PMDD of pGL3-CMV-luciferase and pCIneo-CMV- β -galactosidase was performed at 250 psi using the plate method (8×10^6 cells in $20 \mu\text{l}$) with the delivery device positioned against the base of the tissue culture well directly over the cells. Activity was measured 20 h after PMDD with β -galactosidase results expressed as % of positive cells/total viable cells plated out and luciferase results expressed as cps/ μg protein. For each experiment, data represent mean of triplicate analyses of three transfection suspensions. Broken line joins the means of all experiments on each graph.

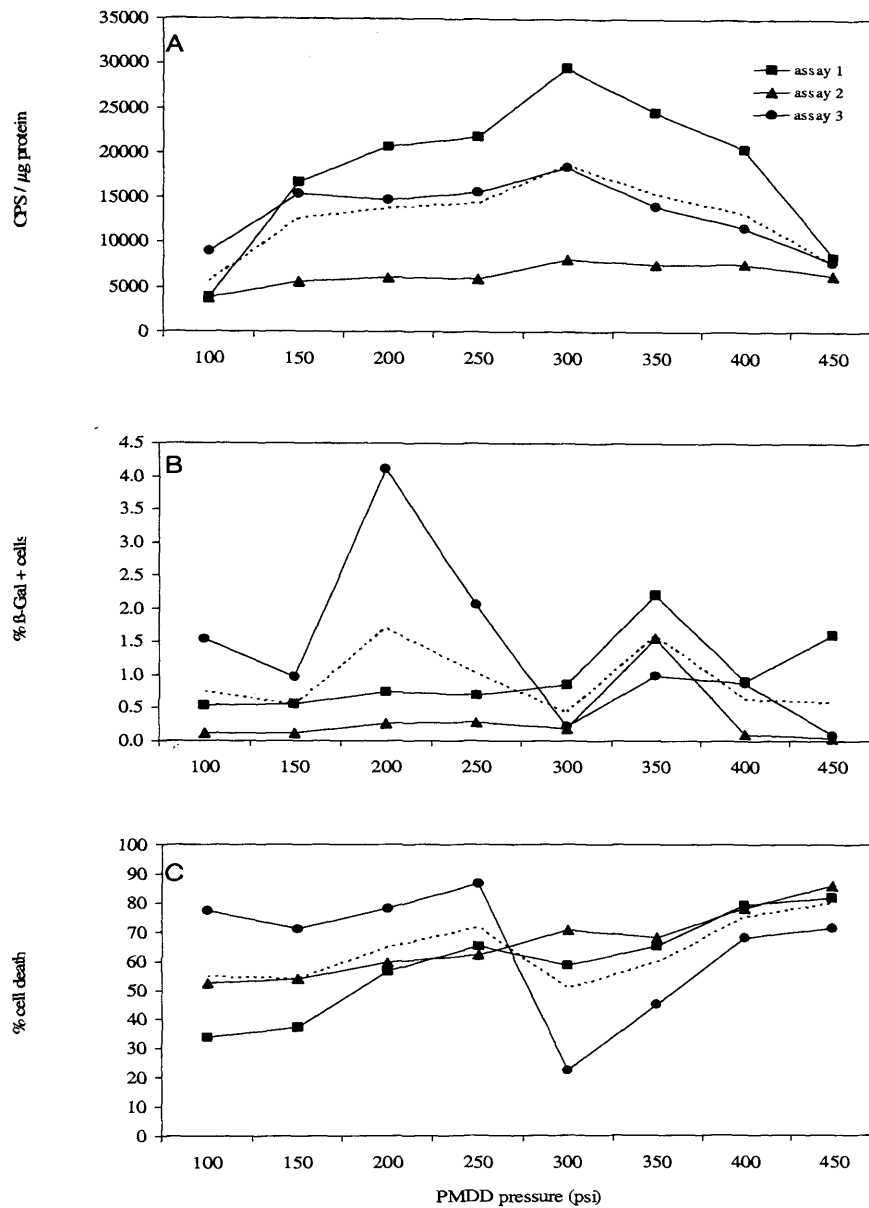


Figure 3.9. The effect of PMDD pressure on luciferase and β -galactosidase transfection efficiency in MC57 cells is shown in panels A and B, respectively. The effect of PMDD pressure on MC57 cell viability following PMDD of β -galactosidase is shown in panel C. PMDD of pGL3-CMV-luciferase and pCIneo-CMV- β -galactosidase was performed using the plate method (8×10^6 cells in $20 \mu\text{l}$) with the delivery device positioned against the base of the tissue culture well directly over the cells. A cell resting time of 6 min was used. Activity was measured 20 h after PMDD with β -galactosidase results expressed as % of positive cells/total viable cells plated out and luciferase results expressed as cps/ μg protein. For each experiment, data represent mean of triplicate analyses of three transfection suspensions. Broken line joins the means of all experiments on each graph.

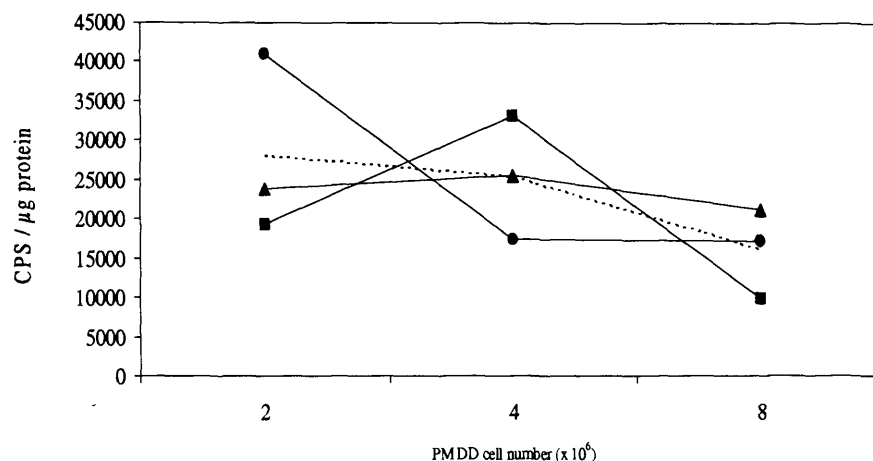


Figure 3.10. The effect of cell concentration on luciferase transfection efficiency. PMDD of pGL3-CMV-luciferase was performed at 300 psi using the plate method ($2-8 \times 10^6$ cells in $20 \mu\text{l}$). Cell resting time of 6 min was used. Activity was measured 20 hrs after PMDD with results expressed as cps/ μg protein. For each experiment, data represent mean of triplicate analyses of three transfection suspensions. Broken line joins the means of all experiments on each graph.

3.4. Discussion

As an emerging technology, PMDD is constantly undergoing modifications to enhance its potential as a delivery system and increase its applications as a transfection tool. Many variants of the delivery device such as those with gun powder, electric charge and helium gas actuation mechanisms have been successfully used in the transfection of mammalian cells. However, this makes it difficult to compare findings between laboratories in terms of optimisation procedures and transfection efficacy of cell lines. Indeed published results demonstrate significant differences in transfection efficiency with different transfection methods (Andreason *et al.*, 1989; Ray *et al.*, 1992). Furthermore, it is evident that certain cell lines such as malignant melanoma B-16 and human breast adenocarcinoma MCF7 (Novaković *et al.*, 1999) are receptive to PMDD while others such as human monocyte derived dendritic cells transfect very poorly or not at all (Smith *et al.*, 2001). Though there is a certain degree of uniformity with respect to transfection conditions used, it is acknowledged that for any one type of delivery device, optimal transfection conditions must be empirically defined for each cell line (Heiser, 1994). In accordance with such findings, transfection of mammalian cells *in vitro* by PMDD using the PowderJect XR1 device in this study appeared to be influenced by both ballistic and non ballistic parameters of the transfection techniques. By using reporter gene assays, it was possible to assess these variables in terms of quality and quantity of transfection.

3.4.1. Influence of ballistic variables

For the suspension method of PMDD, ballistic variables investigated were volume and concentration of cells, PMDD pressure and device positioning. All with the exception of volume of cells proved important. Increasing the concentration of cells and PMDD pressure led to increased transfection efficacy as did positioning the delivery device close to the cell suspension. For the plate method, the effect of concentration of cells, PMDD pressure and cell resting time on transfection efficiency was assessed using MC57s. Results from these studies are not dissimilar to those seen with the suspension method in that increasing

PMDD pressure and cell concentration to a certain point leads to increased transfection efficiency. Beyond this point, transfection efficiency diminishes.

For both methods, there may be reasons why the individual parameters studied affect transfection quality. For example, having a concentrated suspension of cells may create a more viscous target for the DNA coated gold particles, which, when delivered are more successfully retained in the target area leading to increased transfection. Likewise, the distance of the delivery device from the cell suspension determines the target area of the DNA coated gold particles. Thus, the greater the target area and consequent lower density of gold particles is consistent with a lack of improvement in transfection efficiency. Additionally, since the gold particles used are small (2 μM in size), their flight may easily be disrupted by the presence of gasses and particles overlaying the cells. The latter idea is supported by the findings of Klein *et al.* (1987), who observed that increasing the distance between device and target caused an increase in the deceleration force which led to reduced transfection efficiency. However, it is noteworthy that in that particular study, a gun powder charge actuation device was used to accelerate plasmid coated tungsten particles from a cylindrical nylon disc on to target cells. Therefore, although it would be incorrect to assume that the presence of gasses and particles between the device and target cells plays an important role in determining transfection efficiency using the XR1 device, it warrants further investigation.

The variable of greatest importance appears to be PMDD pressure. Increasing pressure has a positive influence on transfection efficiency which may be due to the increasing penetration force of the delivered gold particles. However, results using both methods highlight the importance of achieving a delicate balance between force required for cell penetration by the gold particles and protection offered to the cells. With the plate method, it is evident that exceeding a delivery pressure of 250-300 psi disrupts this balance resulting in decreased cell viability and transfection efficiency. In contrast, it appears as though this balance has not yet been reached when DNA is delivered at 900 psi using the suspension method with little significant difference in cell death between this and lower pressures used. Due to the technical constraint of the pressure regulator, it was not possible to investigate

delivery pressures above 900 psi with the XR1 device. This is unfortunate as many researchers have obtained optimal delivery using PMDD pressures above 900 psi. Fitzpatrick-McElligiot (1992) showed optimal transfection efficiency in Chinese hamster ovary cells and mouse T lymphocyte cell lines (i.e. EL4 and BF-1) at a pressure of 1300 psi. Similarly, Timares *et al.* (1998) demonstrated optimal transfection efficiency of mouse DC (i.e. XS106) and fibroblast (i.e. NS46) cell lines at 950 and 1000 psi, respectively.

In addition to assessing the contribution of individual variables on transfection efficiency, it is also important to consider the relationship that exists between the individual ballistic parameters and the overall effect that this interactivity has on transfection efficiency. For example, the relationship between particle size, target distance and chamber vacuum on particle velocity using a gun powder actuated device was shown by Klein *et al.* (1987). Interestingly, Heiser (1994) demonstrated an interaction between these parameters using a similar device (i.e. using a helium gas and not gun powder actuation mechanism) but suggested that the interactive relationship applied only to a single particle size (i.e. 1.6 μm). Intuitively, one would expect that interactivity of various ballistic parameters may affect transfection efficiency regardless of the type of delivery device used. A possible negative example of this interactive relationship in these studies is the unusually low transfection efficiency obtained when luciferase plasmid was delivered to DAP-3/ICAMs at 500 psi using the suspension method (Figure 3.3, panel A). In each of the three assays performed, the transfection efficiency obtained at this pressure was not in keeping with the general trend of the data (i.e. increasing transfection efficiency with increasing PMDD pressure). The relationship between PMDD pressure, particle size and target distance on particle velocity at this PMDD pressure may be a negative one and may result in poor transfection efficiency.

3.4.2. Influence of non ballistic variables

For both transfection methods, non ballistic variables investigated were cell type transfected and incubation time after PMDD. The reporter gene expression profiles over time of RAWs, DAP-3/ICAMs and MC57s obtained in this study may be representative of

the different biological functions that they perform *in vivo*. Macrophages play a key role in innate immunity performing such functions as phagocytosis and cytokine secretion such that early promoter activity and gene induction may be advantageous. Data obtained for the macrophage cell line in this study (i.e. RAWs) is in agreement with previous findings in which it was demonstrated that murine macrophages transfected by PMDD expressed high levels of transgene after only 4-8 h (Burkholder *et al.*, 1993). Fibroblasts, on the other hand are typical bystander cells which can act as depots for the slow release of antigen in an immune response and therefore sustained gene expression may be considered beneficial. Another important consideration in the determination of optimal incubation time is choice of reporter gene. Tanner *et al.* (1997) showed that the difference between the kinetics of the GFP and luciferase gene expression following PMDD may affect the determination of optimal incubation time. In that study, luciferase protein showed optimal expression at ~12 h after transfection but degraded rapidly after this point while GFP protein remained intact for longer expressing optimally at ~24 h. However, for the MC57s investigated in this study, the luciferase and β -galactosidase reporter genes used for transfection generally showed similar expression profiles over the time-course studied.

Possibly the most influential variable in determining PMDD efficiency is also the one that is least controllable; the cell line transfected. Yang *et al.* (1990) reported variable chloramphenicol acetyltransferase activity in eight different human cell lines of varying origin using a high-voltage electric discharge device. Furthermore, occasion to occasion variability within the same cell line was demonstrated by Novaković *et al.* (1999) who recorded transfection rates of 2-27 % (average of 10.5 %) and 1-11 % (average of 3.9 %) for MCF7 and L929 cell lines, respectively, using a helium gas driven device. However the inter-assay variation observed with the MC57s in these studies was not as large with transfection efficiencies ranging from 1.7-8.3 %. In general, tumour cell lines (i.e. like MC57s) appear receptive to PMDD with reported transfection rates of up to 35 % (Zhang *et al.*, 2002) while dendritic cells (i.e. like BMDCs) transfect very poorly or not at all (Smith *et al.*, 2001; Zhong *et al.*, 1999). These studies as well as the data presented in this chapter support the opinion that, even after process optimisation, it is the intrinsic transfectability of

the cell line itself which may have the greatest influence on determining PMDD transfection rates.

3.5. Conclusions

When the plate and suspension methods of PMDD to mammalian cells were evaluated, the maximum level of reporter gene expression using the plate method exceeded that of the suspension method. Of the cell lines investigated, MC57s transfected the best. EL4 and DAP-3/ICAM displayed only adequate levels of transfection while BMDC proved refractory to transfection by this method. These results demonstrate two important features of this transfection technique.

- The first is that with the possible exception of delivery pressure, optimising experimental variables and transfection methods has relatively little effect on improving the transfection efficacy of a cell line. However, the contribution of certain potentially important variables such as gold particle size and DNA loading has not been evaluated. Also, possible improvements in delivery device design may make these parameters more influential and facilitate improved transfection.
- Secondly and more significantly, large differences in transfection efficiencies between cell lines achieved here and elsewhere infer that it is the intrinsic transfection ability of the cell line itself that ultimately dictates transfection efficacy.

Chapter 4

Development of an *in vitro* CD8+ T cell assay

4.1. Introduction

During the development of DNA vaccines they are tested in prophylactic or therapeutic efficacy models in animals and success in these models is the critical requirement for progression into man. Thus, there is almost a total reliance on *in vivo* studies with *in vitro* or *ex-vivo* methods not yet routine surrogates for *in vivo* vaccine potency. One reason for this may be because it is not clear if the biological markers exploited *in vitro*, such as cytokine production, T cell activation or target cell activity, are true functional surrogates of *in vivo* vaccine efficacy. Secondly, it is not fully understood if various different aspects that underpin the use of gene gun technology *in vivo* such as the importance of DNA dose, method of antigen presentation, the relationship between antigen presented on the APC surface and the magnitude and sensitivity of the resulting T cell response and co-immunisation strategies can be adequately studied *in vitro*.

Along with B lymphocytes, T lymphocytes are the mediators of the adaptive response *in vivo*. The two key elements that characterise their response are specificity and memory. Although having a variety of responsibilities, T cells are generally divided into sub-groups based on their effector function (i.e. Th (CD4+ T cells) or Tc (CD8+ T cells)). CD4+ T cells interact with B lymphocytes helping them to divide and differentiate into either plasma cells or memory B cells. They also interact with macrophages and monocytes allowing these cells to destroy intracellular pathogens. CD8+ T cells recognise and eliminate host cells infected by virus or intracellular pathogens. Exploring the correlation between CD4+ and CD8+ T cell responses *in vivo* and *in vitro* would provide useful information on whether these biological markers, studied *in vitro* are predictive of the *in vivo* immune response. Performing parallel studies *in vitro* and *in vivo* would also allow gene gun immunisation to be dissected into its individual component parts. This would provide a mechanism to assess the relative contribution of each of these component parts to the overall technology.

In this chapter, functional bioassays in which PMDD transfected APCs were used to stimulate antigen specific CD4+ and CD8+ T cell proliferation were developed. The optimal *in vitro* PMDD conditions established in chapter 3 were used for transfections. The

APCs used were those cell lines previously found to be transfectable by PMDD. Additionally, because of their immunological importance and unique ability to activate cellular and humoral responses *in vivo*, BMDCs were also assessed as potential APCs. It is anticipated that such cells, if effectively transfected would present antigen in both the MHC I and II pathways, and could therefore be employed as APCs in both *in vitro* CD8+ and CD4+ T cell assays. Responder cells were MHC matched splenocytes or purified T cells from transgenic mice. Using transgenic mice as sources of responder cells provided a population of specific T cells at a much higher frequency than normal mice, an important consideration when dealing with APC with low transfection efficiency. Responder cells used in the development of these assays were either freshly isolated (i.e. naïve) or cultured for a period in the presence of specific peptide and IL-2 (i.e. activated).

4.2. Objective

- Development and validation of CD8+ and CD4+ *in vitro* activation assays using PMDD transfected APCs.

4.3. Results

The term ‘transfection’ refers to PMDD transfection using the plate method with a cell concentration of 4×10^6 cells/20 μ l and PMDD pressure of 250 psi as optimised in chapter 3. For individual experiments, data is represented as mean \pm standard deviation of triplicate analyses of an individual transfection. Any deviations from this PMDD transfection method or data representation are noted.

4.3.1. *In vitro* CD8+ T cell assay

4.3.1.1. Cell surface marker expression of responder cells

In the *in vitro* CD8+ T cell assay, EL4 and MC57s transfected with pVAC1.NT were investigated as APCs for naïve or activated RAG1.F5 splenocytes. Presenter and responder

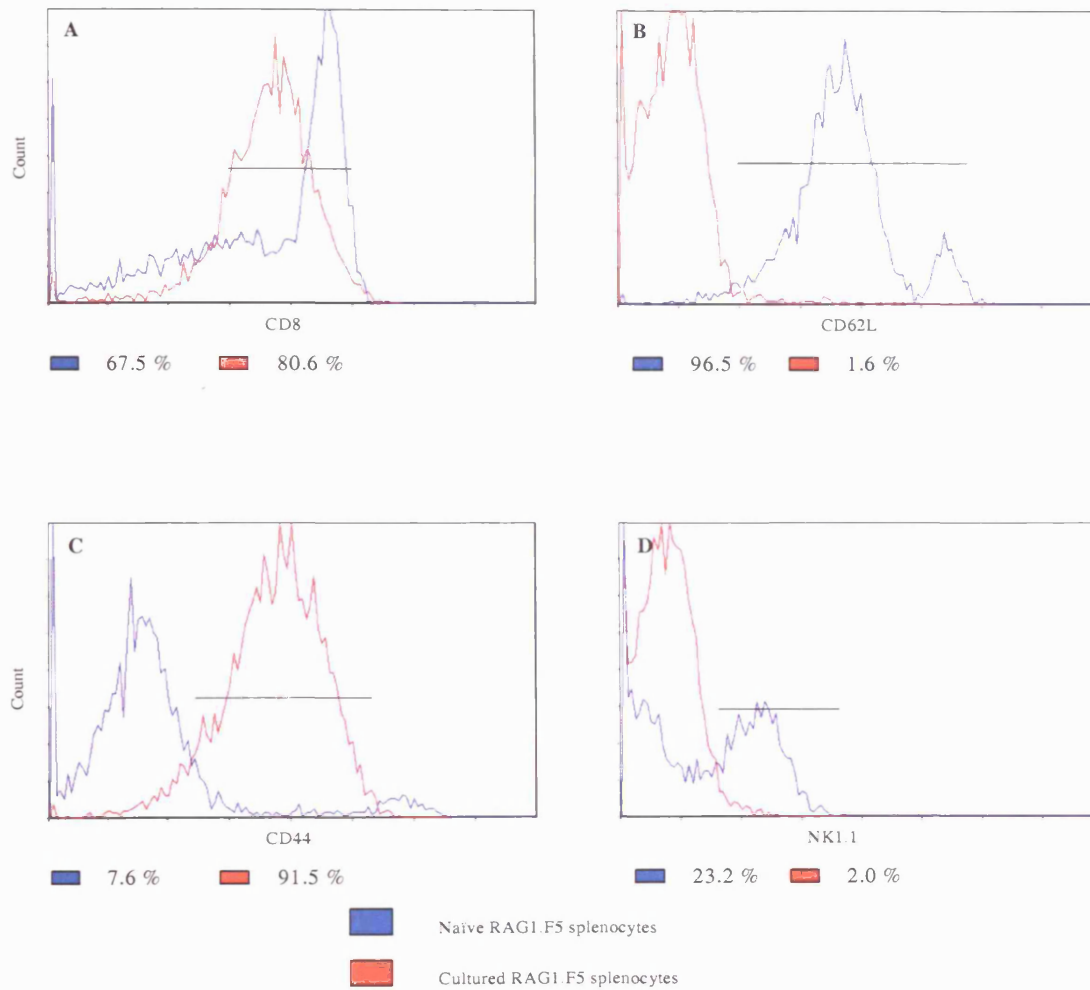


Figure 4.1. Phenotypic analysis of RAG1.F5 splenocytes as assessed by flow cytometry. Naïve RAG1.F5 splenocytes were prepared from the spleens of RAG1.F5 mice on day of harvest. Cultured (activated) RAG1.F5 splenocytes were prepared by culturing naïve splenocytes in RPMI medium containing 500 nM NT peptide (ASNENMDAM) and 20 ng/ml IL-2 for 7 days. Panels A-D are representative fluorescence histograms of CD8, CD62L, CD44 and NK1.1 cell surface expression on naïve (blue) and 7 day *in vitro* cultured splenocytes (red), respectively. Cells in panels B-D were gated on CD8+ splenocytes.

cells were combined in a 1:1 ratio of 1×10^5 cells each with T cell activation measured by IFN- γ ELISPOT. The difference in cell surface marker expression between naïve and activated RAG1.F5 splenocytes is shown in Figure 4.1. When the naïve splenocytes are cultured for 7 days *in vitro* in the presence of NT antigen and IL-2 (i.e. activated splenocytes), their CD8 marker becomes expressed on a greater number of cells but the actual intensity of expression decreases when compared with the naïve freshly isolated RAG1.F5s (panel A). When the naïve splenocytes were gated on their CD8+ population, they exhibited a high level of CD62L expression (e.g. ~96 %) and a low level of CD44 expression (i.e. ~7.6 %). In contrast, activated RAG1.F5 splenocytes when gated on their CD8+ cells expressed very low levels of CD62L (i.e. ~1.6 %) and very high levels of CD44 (i.e. ~92%). Additionally, activated splenocytes were a much purer source of CD8+ T cells with these cells expressing the NK cell marker, NK1.1 at ~2 % compared to the intermediate expression levels observed for naïve splenocytes (i.e. ~23 %).

4.3.1.2. MC57 *in vitro* CD8 T cell assay

The smallest number of spots detectable in the *in vitro* CD8+ T cell assay and the minimum number of APCs that achieved this was determined using A/NT/60/68₃₆₆₋₃₇₄ peptide pulsed MC57 cells as APCs to activated RAG1.F5 splenocytes (Figure 4.2, panels A and B). Cells were pulsed with a range of peptide concentrations (500-1 nM) and added at log dilutions of 1×10^4 to 1×10^1 with 1×10^5 activated splenocytes. Results from panel A revealed that the IFN- γ ELISPOT assay was capable of detecting ≥ 10 cytokine spots (i.e. ~12, ~17, ~19 and ~39 IFN- γ spots were obtained when cells were pulsed with peptide concentrations of 1, 20, 100 and 500 nM, respectively and added at 1×10^2 cells/well). The minimum number of cells that gave an actual readout in the assay was 1×10^2 cells. This was achieved at all peptide concentrations tested in panel A but only at peptide concentrations of 500 and 100 nM in panel B. This suggests that the cells may need to be pulsed with a peptide concentration of ≥ 100 nM before T cell activation occurs consistently, especially at the lower cell numbers. Above the lower limits of the assay (i.e. 1×10^2 APC/well and detection of ≥ 10 spots), the relationship between number of peptide pulsed MC57 cells/well (pulsed with 500 nM peptide) and resulting cytokine spots detected was investigated

(Figure 4.2, panel C). Results show a good correlation between the log of MC57 cells/well and resulting cytokine spots detected with an average R^2 value of 0.99 obtained (mean of 4 assays) over the cell number/well range investigated (1×10^2 - 1×10^4 cells).

Next, the minimum number of PMDD transfected MC57s required to stimulate activated RAG1.F5 splenocytes was established (Figure 4.3). Transfected MC57s were combined in log dilutions from 1×10^5 - 1×10^1 with 1×10^5 activated splenocytes and specific activation measured by number (panels A and C) and area (panels B and D) of IFN- γ spot forming cells on three occasions. Results show that, irrespective of whether the splenocytes came from the same (row A) or different (row B) preparations, the minimum number which yielded a response was 1×10^4 cells. When this number of PMDD transfected cells was used as APC, < 10 resulting cytokine spots were detected (~4 and ~7 spots /well in panels A and C respectively). Therefore, it is likely that $>1 \times 10^4$ transfected MC57s/well (i.e. 1×10^5) are required to get a good level of RAG1.F5 splenocyte stimulation in this assay system. One hundred thousand MC57s/well yielded ~90 cytokine spots regardless of whether the same or different splenocyte preparations were used as responders. Using the β -galactosidase reporter gene, it has previously been established that MC57 PMDD has a transfection efficiency of ~2.5 % (see Figure 3.6). Together, this gives a transfected APC:cytokine spot ratio of 2500:90 (i.e. 2.5 % of $1 \times 10^5 = 2500$). When the peptide pulsed MC57s were used as APC, a corresponding pulsed APC:cytokine spot ratio of 2500:110 was obtained (Figure 4.2, panel C).

4.3.1.3. Choice of antigen presenting cells

EL4 and MC57s were compared for their ability to stimulate naïve RAG1.F5 splenocytes (Figure 4.4). Results for EL4 cells as APC showed no difference between cells transfected with pVAC1.NT and irrelevant plasmid (mean values of ~30 and ~40 IFN- γ spots/well, respectively) indicating no NT specific T cell activation. When MC57s were used as APC, cells transfected with pVAC1.NT yielded a mean value of ~35 IFN- γ spots /well compared to ~17 IFN- γ spots/well for cells transfected with the irrelevant plasmid. However, this

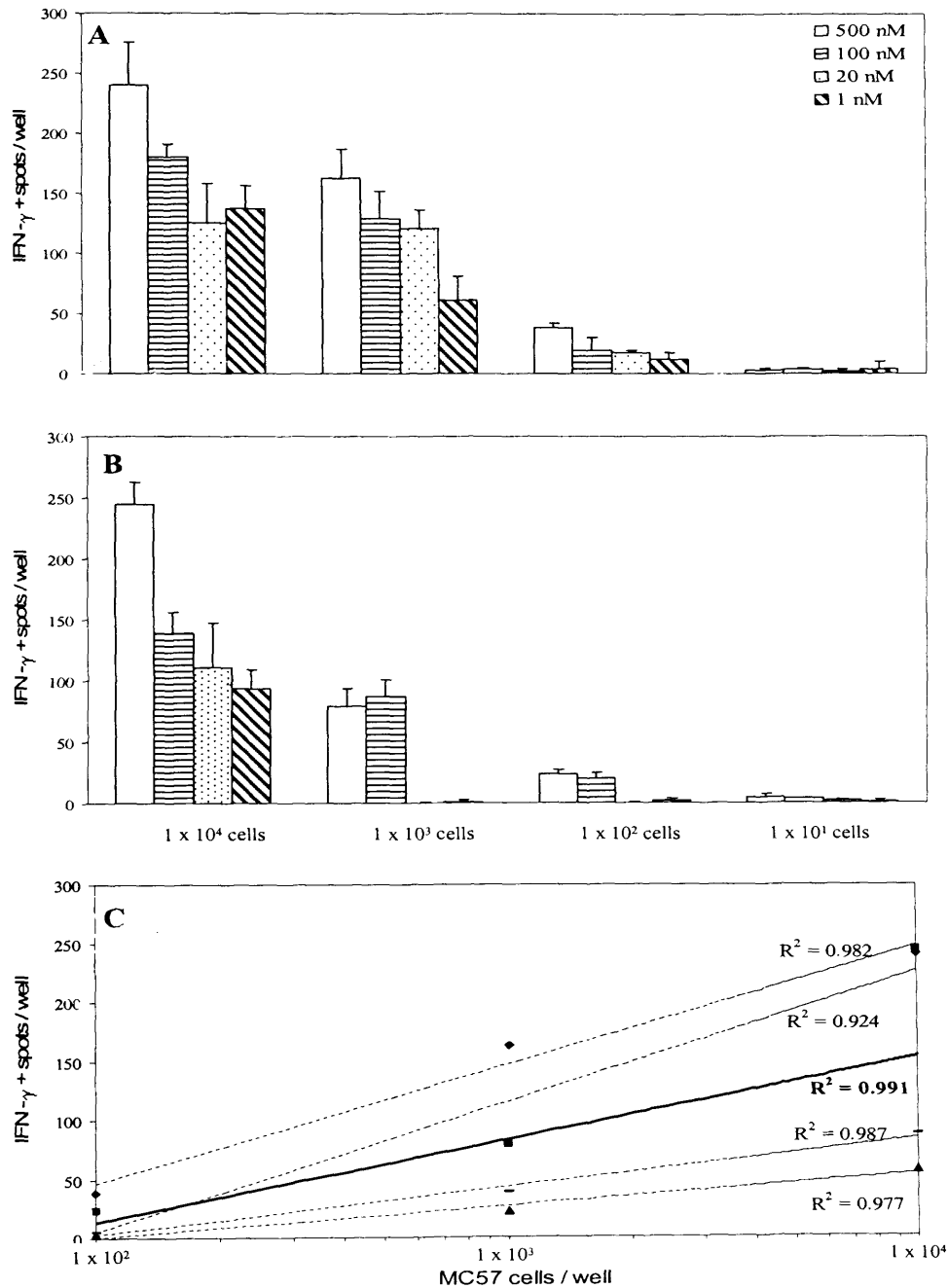


Figure 4.2. Panels A and B: Minimum number of cytokine spots detected in the IFN- γ ELISPOT assay. MC57 cells were pulsed with NT peptide (500-1 nM) and added at log dilutions of 1×10^4 to 1×10^1 with 1×10^5 activated RAG1.F5 splenocytes. Panel C: Relationship between MC57 cells/well and cytokine spots detected. Cells were pulsed with 500 nM NT peptide and added at log dilutions of 1×10^4 to 1×10^2 with 1×10^5 activated RAG1.F5 splenocytes. For A and B, data is from one experiment and is mean \pm standard deviation of triplicate analyses of an individual transfection. For C, each symbol corresponds to an individual experiment with bars showing the mean of all experiments for each group.

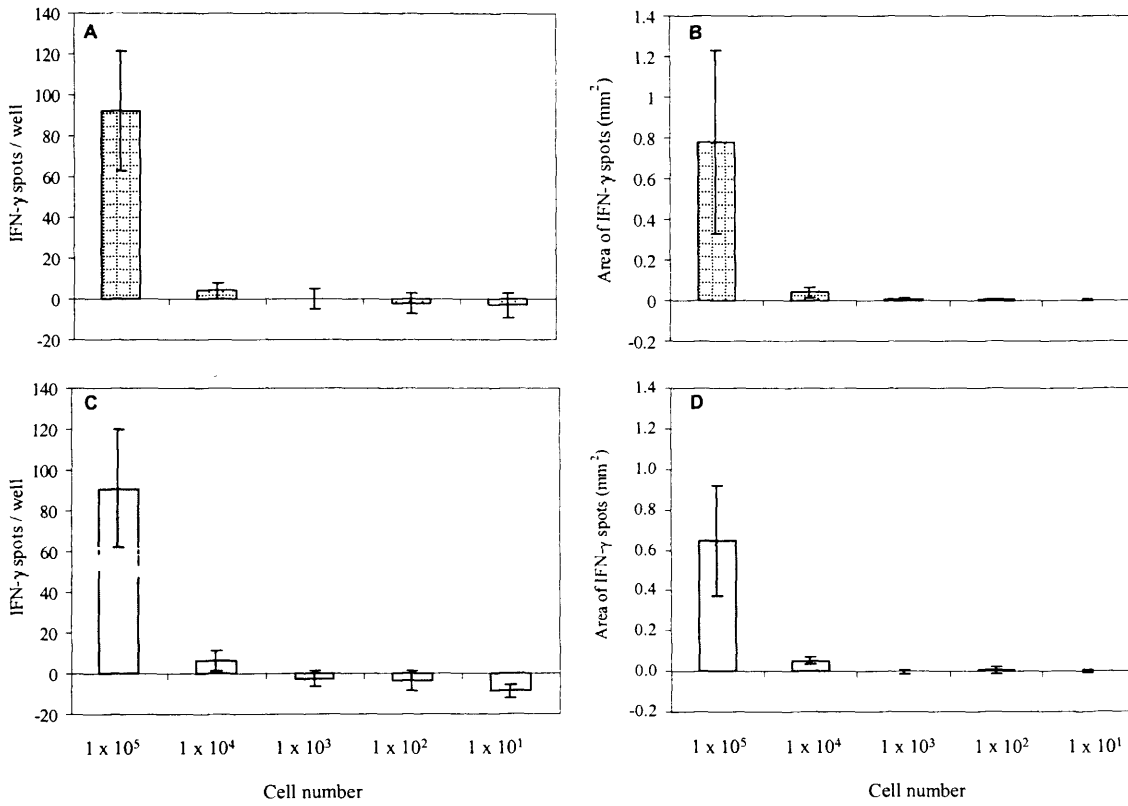


Figure 4.3. Stimulation of activated RAG1.F5 splenocytes by MC57 cells. MC57s were transfected with pVAC1.NT and added at log dilutions of 1×10^5 to 1×10^1 cells with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2. The assay was performed using the same (Row A) or different (Row B) batches of splenocytes and IFN- γ was measured by number (panels A and C) and area (panels B and D) of cytokine positive spots in ELISPOT assays. Each bar represents mean \pm SEM of three individual assays after subtraction of corresponding negative controls groups (i.e. pVAC1.OVAs control groups).

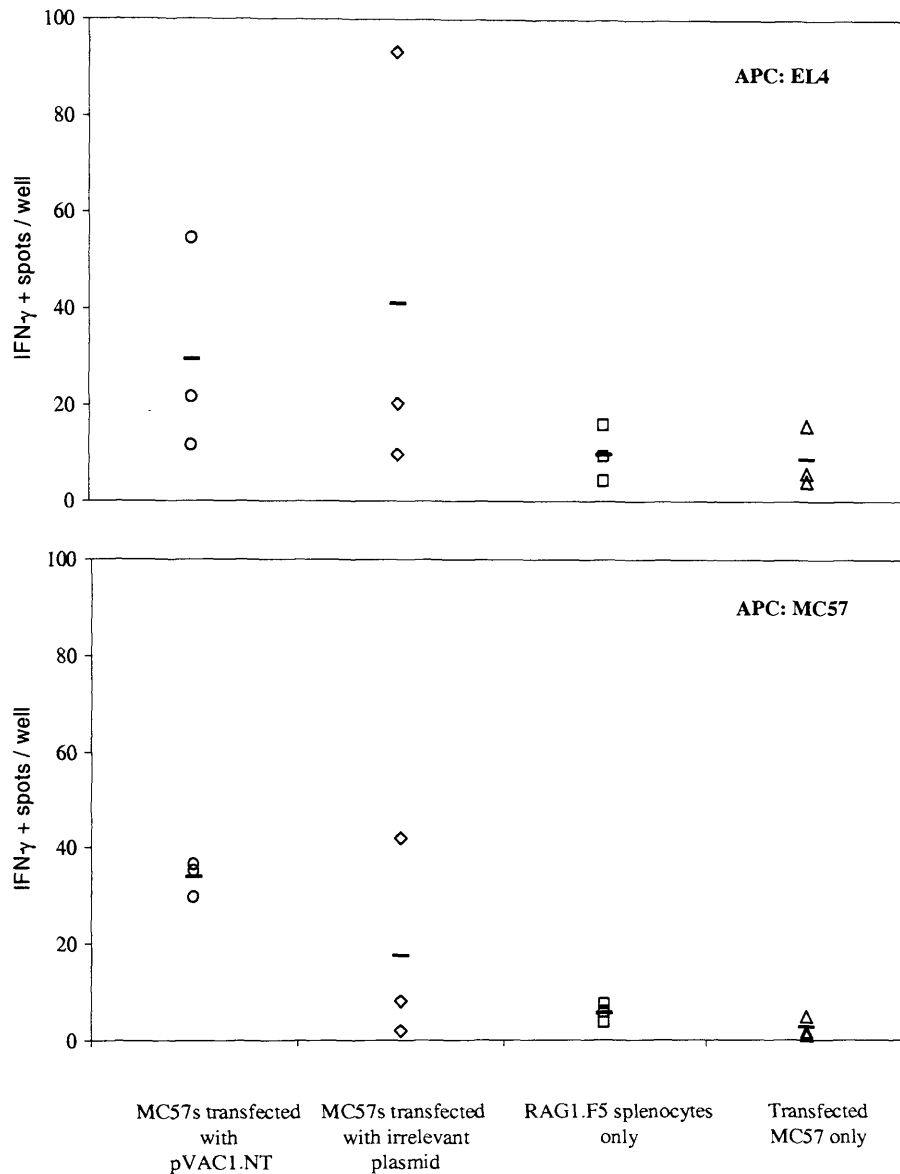


Figure 4.4. Stimulation of naïve RAG1.F5 splenocytes by EL4 and MC57 cells are shown in upper and lower panels, respectively. Cells were transfected with pVAC1.NT or irrelevant plasmid (either pVAC1 or pGL3-CMV-luciferase) and incubated overnight with 1×10^5 naïve RAG1.F5 splenocytes and 200 ng/ml IL-2 before IFN- γ ELISPOT analysis. Groups containing splenocytes and transfected APC only were included as additional negative controls. Each symbol corresponds to an individual experiment with bars showing the mean of all experiments for each group. Student's T-test on EL4 and MC57 group means: pVAC1.NT and irrelevant plasmid group are similar.

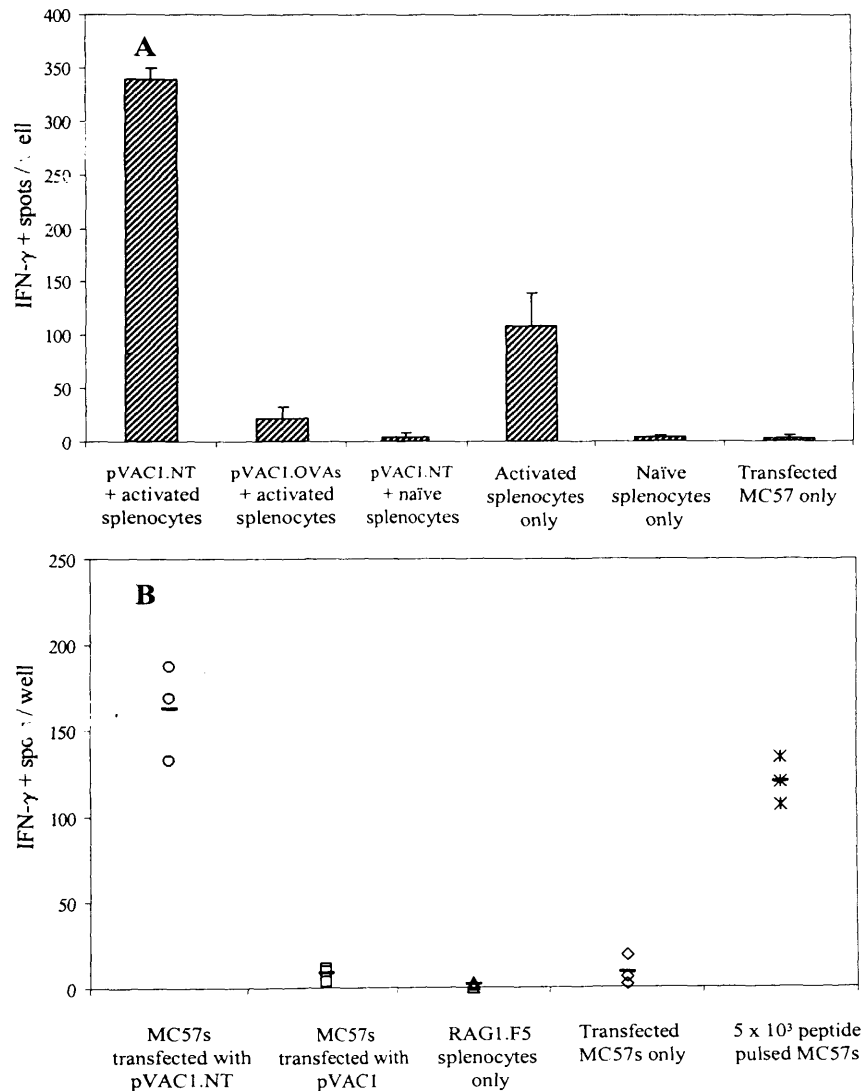


Figure 4.5. Panel A: Stimulation of activated and naïve RAG1.F5 splenocytes by MC57 cells as measured by IFN- γ ELISPOT. MC57 cells were transfected with pVAC1.NT or pVAC1 and incubated overnight with 1×10^5 activated or naïve RAG1.F5 splenocytes and 200 ng/ml IL-2. Groups containing splenocytes and transfected APC only were included as additional negative controls. Data is from one experiment and is mean \pm standard deviation of triplicate analyses of an individual transfection. Panel B: Activation of activated RAG1.F5 splenocytes by MC57 cells measured by IFN- γ ELISPOT. MC57s were transfected with pVAC1.NT or pVAC1 and incubated overnight with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2. Positive control was 5×10^3 MC57 cells pulsed with 500 nM NT peptide. Groups containing RAG1.F5 splenocytes and transfected MC57s only were included as additional negative controls. Each symbol corresponds to an individual experiment with bars showing the mean of all experiments for each group. Student's T-test on panel B: the pVAC1.NT and pVAC1 groups are significantly different ($p < 0.001$).

result did not achieve significance due to high value obtained for the irrelevant plasmid group in one of the three assays performed (~40 IFN- γ spots/well). When this assay was removed from the analysis, there was then a noticeable difference between the positive and negative PMDD transfected groups. For both cell types, the control groups of RAG1.F5 splenocytes only, or transfected APCs only, gave similar background levels of T cell activation as the group transfected with irrelevant plasmid (in two of the three assays).

4.3.1.4. Activation state of responder cells

Using transfected MC57 as APC, naïve and activated RAG1.F5 splenocytes were directly compared as responders in the CD8+ *in vitro* assay (Figure 4.5, panel A). Results revealed that the activated splenocytes responded much more efficiently than the naïve splenocytes (~340 compared with ~5 IFN- γ spots/well). Secondly, when activated RAG1.F5 splenocytes were used as responders, there was a 15 fold difference in NT specific T cell activation between the MC57 groups transfected with pVAC1.NT and pVAC1.OVAs (control group) (i.e. ~340 and ~22 IFN- γ spots/well, respectively). Activated splenocytes as responders to pVAC1.NT transfected MC57s were further investigated in Figure 4.5, panel B. These transfections were performed using a PMDD pressure of 275 psi, resting time of 6 min and incubation time after PMDD of 20 hrs. Individual experiments represents mean of triplicate analyses of the mean of three transfections. Results indicated specific T cell activation with pVAC1.NT and pVAC1 transfected APCs gave ~1625 and ~10 IFN- γ spots/well, respectively. A surprising observation from the data shown in Figure 4.4 was the unusually high level of IFN- γ secretion associated with activated splenocytes only in panel A (~110 IFN- γ spots/well). This result was from an individual assay and was not in accordance with the similar group shown in panel B which yielded a mean value of only ~2 IFN- γ spots/well (three assays performed).

4.3.2. *In vitro* CD4+ T cell assay

DAP-3/ICAM cells transfected with pVAC1.OVAs were used as APCs to DO.11.10 splenocytes or CD4+ T cells isolated from these splenocytes. Presenter and responder cells

were combined in a 1:1 ratio (2×10^5 cells each). Production of IL-2 (indicating specific T cell proliferation) as measured by ELISA and ELISPOT, and T cell proliferation were used to measure activation.

The minimum number of OVA₃₂₃₋₃₂₉ peptide pulsed DAP-3/ICAM cells required to stimulate naïve CD4+ DO.11.10 T cells was investigated (Figure 4.6). Results showed that if the IL-2 ELISA and T cell proliferation assays were used to measure activation, at least 1×10^3 DAP-3/ICAMs/well were required for T cell stimulation. If the IL-2 ELISPOT assay was used, only 1×10^2 DAP-3/ICAMs/well were required. Using the β -galactosidase reporter gene, it was shown that DAP-3/ICAMs had a PMDD transfection efficiency of ~0.8 % (see Figure 3.5). Together, this predicts that 2×10^5 transfected DAP-3/ICAMs would generate a detectable response in all three readout assays, especially the IL-2 ELISPOT (i.e. 0.8 % of 2×10^5 is 1.6×10^3 cells). This prediction is based on the assumption that each transfected DAP-3/ICAM cell can effectively produce, process and present the synthesised antigen.

DAP-3/ICAM cells transfected with pVAC1.OVAs were assessed for their ability to stimulate naïve DO.11.10 CD4+ T cells or activated DO.11.10 splenocytes. When the naïve DO.11.10 CD4+ T cells were used as responders (Figure 4.7), no specific T cell activation was evident in any of the readout assays with both transfected and non-transfected DAP-3/ICAM groups showing approximately equal levels of T cell activity. It is also clear from the T cell proliferation and IL-2 ELISPOT data that DAP-3/ICAM cells pulsed with OVA peptide yielded a noticeably stronger IL-2 response than did cells pulsed with whole protein (i.e. 3.2 and 1.4 fold increases in T cell activity in the proliferation and ELISPOT assays, respectively). DAP-3/ICAM cells pulsed with whole protein did not produce a detectable response in the IL2-ELISA. A similar result was obtained when activated DO.11.10 splenocytes were employed as responders in an IL-2 ELISPOT assay (Figure 4.8). No detectable CD4+ T cell specific activation was observed with the pVAC1.OVAs and pVAC1 transfected DAP-3/ICAM groups generating a similar response.

4.3.3. T cell stimulation by PMDD transfected dendritic cells

The ability of BMDCs to stimulate T cell activity is shown in Figure 4.9. BMDCs derived from DO.11.10 and BALB/c mice were transfected with pVAC1.OVAs and incubated with purified naïve DO.11.10 CD4+ T cells. Consistent with the findings from the reporter gene studies (section 3.3.3), the overall results revealed that no specific T cell activation was obtained compared with non transfected BMDCs. Actually, IL-2 secretion in the non-transfected population was greater than that of BMDCs transfected with pVAC1.OVA (all had a seeding density of 4×10^4 cells/well) regardless of the type of BMDCs transfected. This result may indicate possible deleterious effects of the PMDD transfection procedure on this type of BMDCs.

4.4. Discussion

In order to establish *in vitro* functional assays, different PMDD transfected cell lines were investigated as APCs to DO.11.10 and RAG1.F5 responder cells in a number of *in vitro* experimental systems. In each case, successful activation was dependent on a number of factors. These included efficiency of PMDD transfection of the APCs, sensitivity of the assay system used, maturation state of the APCs and responder cells and whether the synthesised peptide was presented through the correct pathway.

4.4.1. Efficacy of PMDD transfection of antigen presenting cells

The stimulatory potential of each APC appeared to be proportional to the amount of synthesised gene product within the cells themselves. Transfection efficiency of all of the cell lines was <1 % except for MC57s which transfected at an efficiency of ~2.5 %. These transfection levels were reflected in MC57s ability to stimulate naïve RAG1.F5 splenocytes compared with EL4s. Likewise, EL4s and DAP-3/ICAMs transfected with similar efficiency by PMDD and both cell lines demonstrated no capacity to stimulate responder cells. This finding may suggest that for non professional APCs at least, the most influential

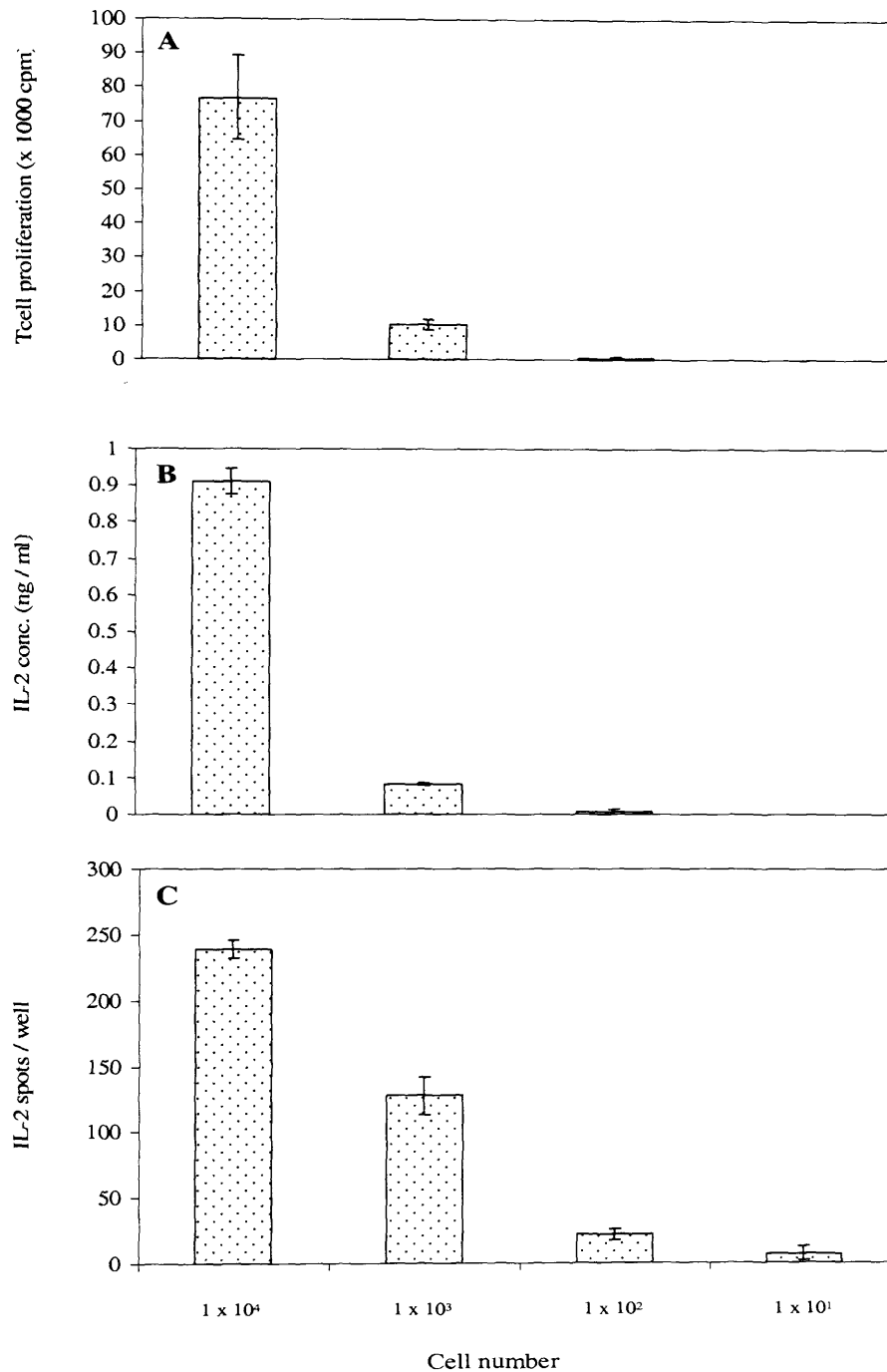


Figure 4.6. Minimum number of APC which gave a detectable response in the *in vitro* CD4+ T cell assay as measured by T cell proliferation (A), ELISA (B) and ELISPOT (C). DAP-3/ICAM cells (pulsed with 100 μ M OVA₃₂₃₋₃₂₉ peptide) at log dilutions of 1×10^4 to 1×10^1 were incubated overnight with 2×10^5 naïve DO.11.10 CD4+ T cells and IL-2. For each assay, data is from one experiment and is mean (\pm standard deviation) of triplicate analyses of an individual transfection.

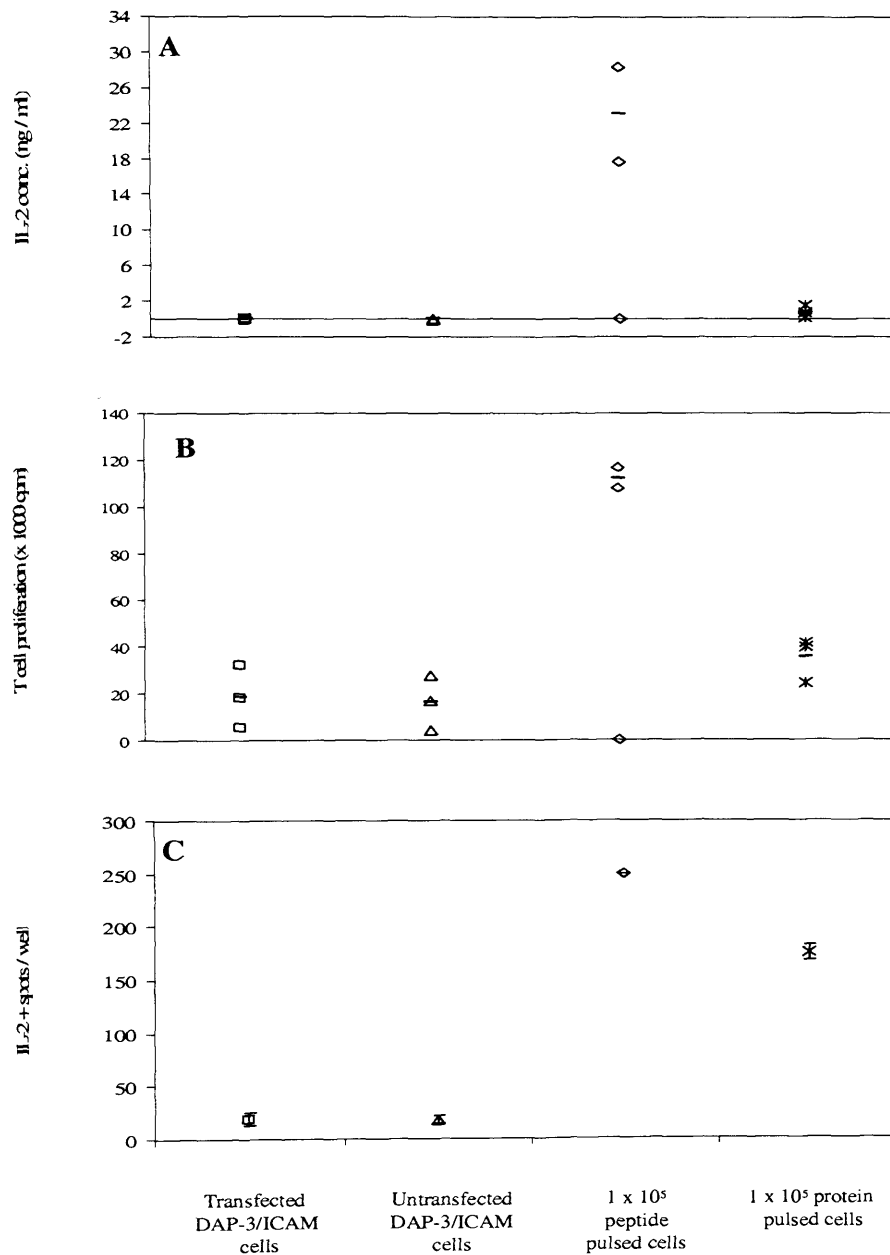


Figure 4.7. Activation of naïve DO.11.10 CD4+ T cells by DAP-3/ICAM cells. IL-2 secretion was measured by ELISA (A), T cell proliferation (B) and ELISPOT (C). Transfected or non-transfected DAP-3/ICAMs (2×10^5 cells) were incubated overnight with 2×10^5 naïve DO.11.10 CD4+ T cells prior to analysis. Positive controls were 1×10^5 DAP-3/ICAM cells pulsed with $1 \mu\text{M}$ OVA₃₂₃₋₃₂₉ peptide or OVA protein. In panels A and B, each symbol corresponds to an individual experiment with bars showing the mean of all experiments for each group. In panel C, each symbol is one experiment and is mean (\pm standard deviation) of triplicate analyses of an individual transfection.

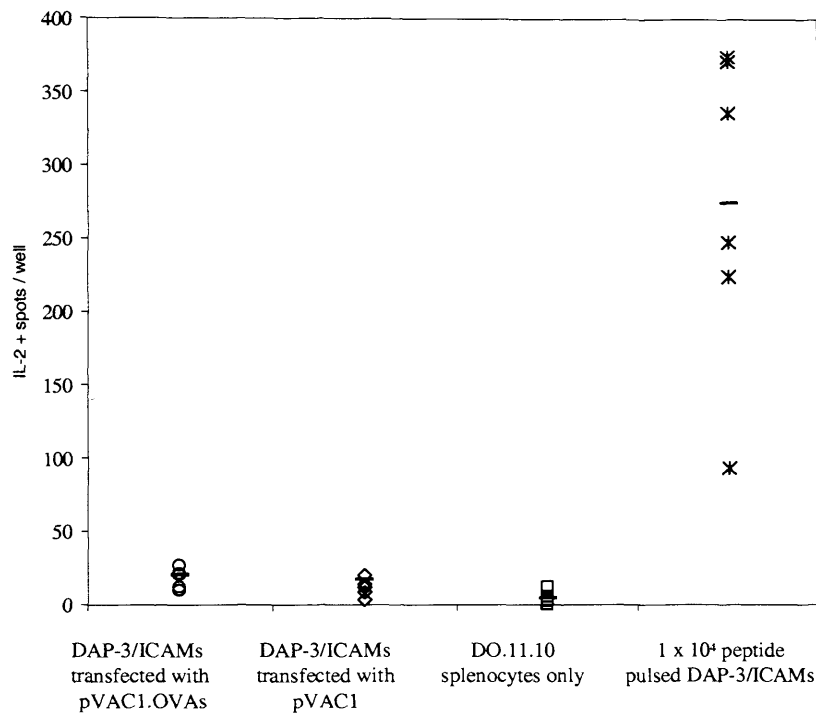


Figure 4.8. Stimulation of activated DO.11.10 splenocytes by DAP-3/ICAM cells measured by IL-2 ELISPOT. DAP-3/ICAMs (2×10^5 cells) were transfected with pVAC1.OVAs or pVAC1 and incubated overnight with 2×10^5 activated DO.11.10 splenocytes. Positive control was 1×10^4 DAP-3/ICAM cells pulsed with $1 \mu\text{M}$ OVA₃₂₃₋₃₂₉ peptide. A group containing DO.11.10 splenocytes only was included as an additional negative control. Each symbol corresponds to an individual experiment with bars representing the mean of all experiments for each group.

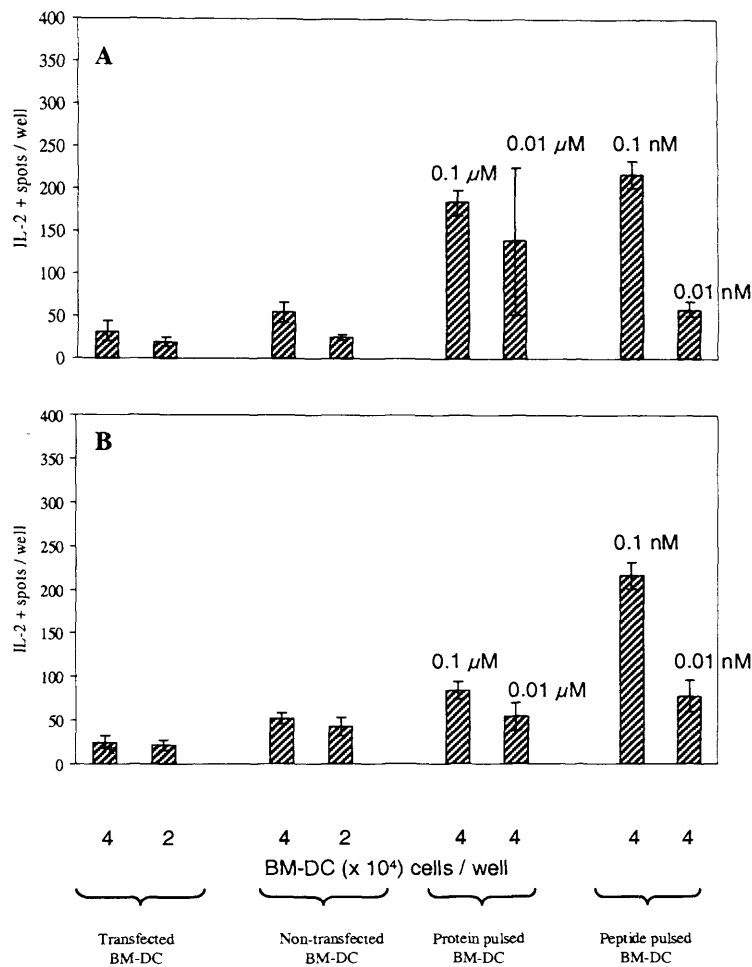


Figure 4.9. Activation of naïve DO.11.10 CD4+ T cells by BMDC measured by IL-2 ELISPOT. Panels A and B are DO.11.10 and BALB/c BMDC transfected with pVAC1.OVAs, respectively. Positive controls were 4 x 10⁴ BMDC pulsed with OVA protein (0.1 and 0.01 μ M) or OVA₃₂₃₋₃₂₉ peptide (0.1 and 0.01nM). Transfected BMDC, non-transfected BMDC and positive controls were incubated overnight with 2 x 10⁵ DO.11.10 CD4+ T cells. For each type of BMDC transfected, data is from one experiment and is mean (\pm standard deviation) of triplicate analyses of an individual transfection.

factor in influencing T cell stimulatory capacity is the transfection efficiency of the APC and that once a certain threshold level of transfection is reached (as in the case of MC57s), then the APC has the capacity to stimulate T cell activity. DCs are the most potent APC for inducing a T cell immune response *in vivo* and are capable of MHC class I and class II antigen presentation (Banchereau and Steinman, 1998). For these cells, particle mediated transfection efficacy *in vitro* appears to be dependent on the type of DC precursor used. With human DCs, cells derived from CD34+ hematopoietic progenitors which divide in culture (e.g. BMDCs) have been shown not be transfected by PMDD (Lundqvist *et al.*, 2002). For non dividing cells, such as monocyte derived DCs, some investigators have shown successful transfection by PMDD (Tuting *et al.*, 1998) while others have not (Smith *et al.*, 2001; Zhong *et al.*, 1999). In contrast, murine DCs, whether dividing or non-dividing, prove totally refractory to PMDD with the only reported exception been the transfection of a DC line which exhibited the phenotypic and functional characteristics of mature Langerhans cells (Timares *et al.*, 1998). In that particular study, the percentage of GFP-positive cells was in the range of 0.1-0.6 % with a mean transfection efficiency of 0.25 %. Results from the reporter gene and functional studies described in this chapter concur with the overall view that murine DC are non-transfectable and this most likely explains the failure of pVAC1.OVA transfected BMDCs to stimulate DO.11.10 CD4+ T cells.

4.4.2. Sensitivity of immuno-assays

The sensitivities of the readout assays for the *in vitro* functional assays were examined in order to determine whether these limited the ability to detect T cell activation. For the class I *in vitro* assay, the sensitivity of the IFN- γ ELISPOT readout assay was established using peptide pulsed and PMDD transfected MC57s as presenters to activated RAG1.F5 splenocytes. For the peptide pulsed MC57s, the smallest number of spots detectable and the number of APC that gave these spots was calculated to be $\sim 1 \times 10^1$ and 1×10^2 , respectively. When PMDD transfected MC57s were used, the smallest number of spots detectable was $< 1 \times 10^1$ and the number of APC that gave these spots was 1×10^4 . Therefore, as expected, positive responses were obtained when 1×10^5 PMDD transfected

MC57s were used as APCs to naïve and activated RAG1.F5 splenocytes. Above the limits of the assay as established by peptide pulsed MC57s, there was a relationship between the numbers of APC/well and resulting cytokine spots formed. This relationship was semi-logarithmic and not linear (Figure 4.2, panel C) with the APC:cytokine spot ratio very high (e.g. when 1×10^3 peptide pulsed cells/well were used, the APC: cytokine spot ratio was 1000:70). Theoretically, the expectation is that one APC with intact MHC I/NT peptide complexes present on its surface should be sufficient to stimulate T cell activity (i.e. a theoretical APC:cytokine spot ratio of 1:1), yet this clearly did not happen. One possibility for this may be the lack of responsiveness of the activated RAG1.F5 splenocytes. The *in vitro* culturing process (i.e. in the presence of IL-2 and NT peptide) was important for increasing the responsiveness of the splenocytes relative to naïve, freshly isolated splenocytes but may have left the cells vulnerable to AICD when re-exposed to peptide laden APC in the *in vitro* assay. Furthermore, there were no CD4+ helper cells present in the *in vitro* system which may have served to support the activity of and maintain the activated RAG1.F5 responders for the duration of the assay. This notion is supported by the data obtained when PMDD transfected MC57s were used as APC. When 1×10^5 MC57 cells/well were used in the assay and adjusted for a PMDD transfection rate of ~2.5 %, an APC:cytokine spot ratio of 2500:90 was obtained. When the same number of peptide pulsed cells were used as APC, a ratio of 2500:110 was obtained. The similarity between these ratios may suggest that it is the behaviour of the responders (although in vast excess compared with the number of APC present in the assay) that are responsible for the high APC:resulting cytokine spot ratio. The similarity between the ratios also suggests that the MC57 cells when transfected by PMDD are proficient at protein synthesis and peptide processing and presentation. An alternative explanation for the high APC:resulting cytokine spot ratio may lie with the MC57 cells themselves. It is possible that these cells are heterogeneous in their ability to present antigen and this would have the effect of increasing this ratio.

For the class II assay, the sensitivity of the three readout assays was established using peptide pulsed DAP-3/ICAMs as presenters to naïve D0.11.10 CD4+ T cells. ELISA and T cell proliferation assays required $\geq 1 \times 10^3$ peptide pulsed APCs in order to obtain a positive

response. For IL-2 ELISPOT, this was achieved with ten fold less APCs. Therefore, assuming that protein synthesis and peptide processing and presentation within the APCs are efficient and taking into account the DAP-3/ICAM PMDD transfection rate, then each assay, and especially ELISPOT, should be sensitive enough to detect specific T cell activation after PMDD. However, no specific activation was obtained with either naïve or activated responders suggesting that inefficient and/or incorrect processing and presentation may be the limiting parameters of these assays (see section 4.4.4).

4.4.3. Activation state of antigen presenting and responder cells

For BMDCs, the degree of maturation may be important in determining its *in vivo* and *in vitro* T cell stimulatory potential (Barratt-Boyes *et al.*, 2000; Labeur *et al.*, 1999). There is evidence that indicates that mature DCs transfect *in vitro* with a greater efficiency than immature DCs (Miller *et al.*, 2003). Although the DCs used in the study were not matured prior to transfection, this may not have been critical, as the physical process of PMDD results in DC maturation and leads to increased expression of cell surface MHC and co-stimulatory molecules as well as increased cytokine secretion (Smith *et al.*, 2001). However, results showed more IL-2 secretion associated with the non-transfected populations than the transfected populations. This suggests that possibly the positive effects of PMDD in increasing DC maturation *in vitro* were outweighed by the deleterious effect of the technique on cell viability. The state of activation of non-professional APCs does not appear to be as critical to their immuno-stimulatory capacity. When MC57s pre-treated with IFN- γ were used as APC in the *in vitro* CD8+ T cell assay, a much larger NT response was obtained compared with untreated MC57s as APC (see Figure 6.9). However, specific T cell activation (i.e. relative difference between positive and negative groups) was approximately equivalent suggesting that the effect of IFN- γ pre-treatment was predominantly non specific.

Increasing the responsiveness of responder cells proved important. Culturing naïve RAG1.F5 splenocytes in the presence of IL-2 and specific peptide resulted in a more active phenotype typified by increased CD8 and CD44 and decreased NK1.1 and CD62L

expression profiles. The difference in responsiveness between naïve and activated RAG1.F5 splenocytes (calculated to be ~85 fold) is more than likely due to the fact that activated splenocytes behave like a memory T cell population (having come in contact with specific peptide during the culturing process). As such, they require fewer MHC/peptide complexes on the APC surface and are less dependent on the presence of the co-stimulation signal on the APC surface for activation to occur.

Separately, when naïve purified CD8+ T cells were used as responders instead of naïve RAG1.F5 splenocytes, peptide pulsed MC57s could only weakly stimulate antigen specific IFN- γ (data not shown). This may be because the MC57s lack co-stimulatory molecules (CD80 and CD86) found on professional APCs. In contrast, endogenous APC present within the splenocyte population which once activated may be able to mature, upregulate costimulatory molecules and decrease the threshold required for CD8+ T cell activation (Ruedl *et al.*, 1999). With this in mind, it would be interesting to combine naïve and activated RAG1.F5 splenocytes as a responder population in an *in vitro* assay and compare this mixed population with naïve and activated splenocytes used separately as responders to PMDD MC57 cells. Having a certain proportion of naïve splenocytes present in such a population may compensate for loss of endogenous APCs due to the culturing process while having activated splenocytes present retains the responsiveness of the activated only splenocyte population.

4.4.4. Providing correct T cell receptor specificity

All of the *in vitro* systems investigated share the requirement of providing correct TcR specificity if responder cell activation is to occur. Direct transfer of plasmid to APCs (as in the case of the CD8+ and CD4+ *in vitro* assays) mainly results in endogenous synthesis of the resulting antigen and subsequent processing and presentation through the class I pathway. This was clearly demonstrated in the CD8+ T cell *in vitro* assay where direct transfection of MC57s with pVAC1.NT resulted in the presentation of NT peptide/MHC I complex in a manner which led to the stimulation of RAG1.F5 splenocytes. The need for a particular TcR specificity may explain why BMDC and particularly, DAP-3/ICAMs failed to stimulate D0.11.10 effector cells. After direct PMDD of DAP-3/ICAMs with

pVAC1.OVAs, ovalbumin is primarily produced and presented in the context of MHC class I hence not providing the correct specificity for the CD4 TcR. The ovalbumin plasmid used in these experiments contained a secretory signal thus allowing it, in theory to access the class II loading compartment. *In vivo*, endogenous APCs resident in the immune microenvironment may capture and process secreted peptide through their exogenous pathway. However, in this *in vitro* system, there may be no other efficient presenters (only DAP-3/ICAMs and DO.11.10 CD4+ T cells) thus attenuating CD4 activation via this route.

This explanation does not hold true for BMDCs for two reasons. Firstly, unlike DAP-3/ICAMs, other bystander BMDCs as professional APCs are capable of capturing secreted peptide and processing it through their exogenous pathway. Secondly, directly transfected DCs can cross present endogenously synthesised peptide thus opening up the class II pathway (Malnati *et al.*, 1992; Sloan *et al.*, 2002; Weissman *et al.*, 2000). Therefore, the most likely reason for the failure of BMDC to stimulate DO.11.10 T cell activity is that these cells are totally refractive to transfection by PMDD.

4.5. Conclusions

In this chapter, *in vitro* functional assays in which PMDD transfected APCs were used to stimulate antigen specific CD4+ and CD8+ T cell proliferation were investigated. The main observations from this work were that

- A major factor in determining successful T cell activation was PMDD transfection efficiency. Transfecting APCs at a level below the sensitivity of the readout assay resulted in the failure to detect an antigen specific response.
- Manipulation of the activation state of the responder cells greatly improved assay sensitivity.
- The requirement for correct TcR specificity was essential in generating a working assay.

Development and validation of an *in vitro* functional assay for CD4+ T cell activation was not achieved, at least partly because of poor PMDD transfection efficiencies. However, a robust *in vitro* functional assay for assessment of CD8+ T cell activation by transfected APCs has been developed and validated using PMDD transfected MC57 cells and T cells from RAG1.F5 transgenic mice. This *in vitro* functional assay is used to investigate in following chapters, the correlation between *in vitro* and *in vivo* immune responses.

Chapter 5

Effect of antigen dose on PMDD efficacy following single plasmid immunisation

5.1. Introduction

Varying the amount of NT antigen available for presentation and studying its effect on resulting immune response provides a way to assess features of gene gun technology such as the importance of DNA dose and the relationship between presented antigen and resulting immune response. In this chapter, both these aspects are studied *in vitro* using the CD8+ T cell assay described in chapter 4 (i.e. pVAC1.NT PMDD transfected MC57s were used as APC to activated RAG1.F5 splenocytes) and *in vivo* using IFN- γ ELISPOT and intracellular IFN- γ staining analysis. By comparing both sets of data, the ability of the *in vitro* CD8+ T cell assay to predict *in vivo* responses is assessed.

In order to do this, the amount of NT antigen made available for presentation was varied in two different ways. Firstly, the amount of NT encoding plasmid delivered by PMDD was varied (i.e. immunisation dose responses). The effects of such dose responses were examined in parallel *in vitro* and *in vivo* experiments. From a quantitative perspective, the relationship between amount of DNA administered by PMDD and elicitation of the CD8+ response has been much studied. Nanogram quantities of nucleoprotein and haemagglutinin encoding DNA delivered intradermally are sufficient to raise cellular responses *in vivo* (Fynan *et al.*, 1993; Pertmer *et al.*, 1995; Degano *et al.*, 1998). For this type of dose response, DNA cartridges containing variable amounts of pVAC1.NT were mixed with an “empty plasmid” (i.e. pVAC1). This ensured that the total amount of DNA delivered by PMDD was constant at 500 ng/cartridge.

Secondly, the amount of intracellular NT protein produced was manipulated at the transcriptional level. Plasmids with different promoters were created for this purpose (i.e. the p7313 vectors) based on the CMV immediate-early (IE) promoter. This constitutive promoter has, in various combinations, been shown to drive high gene expression in transfected cultured cells (Xu *et al.*, 2001; Foecking and Hofstetter, 1986; Boshart *et al.*, 1985) and in multiple tissues in transgenic mice (Schmidt *et al.*, 1990). Among a number of viral promoters tested, the CMV-IE promoter consistently resulted in the greatest amount of

gene expression in human prostate cancer cell lines transfected by PMDD (Zhang *et al.*, 2002).

Of the various additional components introduced to form the different promoters, the CMV-IE enhancer and intron A DNA sequences are the most important. In all constructs, the CMV-IE enhancer was positioned immediately upstream of the transcriptional start site. It is a highly complex regulatory region containing sequences which bind to host encoded transcriptional effector proteins allowing them to activate or repress gene transcription. They function by increasing the probability of stable promoter/transcription factor complexes which result in a greater number of cells expressing a transcriptionally active form of the promoter (Walters *et al.*, 1995) and lead to an increased amount of active RNA polymerases present at these transcriptional start sites (Weber and Schaffner, 1985). Intron A, the largest intron of the CMV-IE 1 gene has been demonstrated to enhance CMV driven gene expression *in vitro* (Niwa *et al.*, 1990; Chapman *et al.*, 1991; Xu *et al.*, 2001). Improved expression is attributed to an increased level of RNA polyadenylation and/or nuclear transport related with RNA splicing (Huang and Gorman, 1990). The effect of these plasmids on intracellular NT production and resulting CD8+ T cell responses were examined *in vitro* and *in vivo*.

5.2. Objective

The importance of DNA dose and the relationship between presented antigen and resulting immune response in gene gun vaccination was investigated by assessing *in vitro* and *in vivo*

- the effect of immunising DNA dose on resulting CD8+ immune responses.
- the effect of a NT promoter dependent dose response on resulting CD8+ immune responses.

The *in vitro* and *in vivo* data were directly compared and the ability of the *in vitro* CD8+ T cell assay to predict *in vivo* responses was assessed.

Cartridge composition		
Group name	Theoretical loading pVAC1.NT ng DNA / cartridge	Theoretical loading pVAC1 ng DNA / cartridge
a	500	0
b	50	450
c	5	495
d	0.5	499.5
e	0.05	499.95
f	0.005	4999.995
g	0	500

Table 5.1. Nomenclature used to denote amount of influenza nucleoprotein encoding plasmid DNA and control plasmid DNA in cartridge preparations for all *in vitro* and *in vivo* experiments presented in chapter 5.

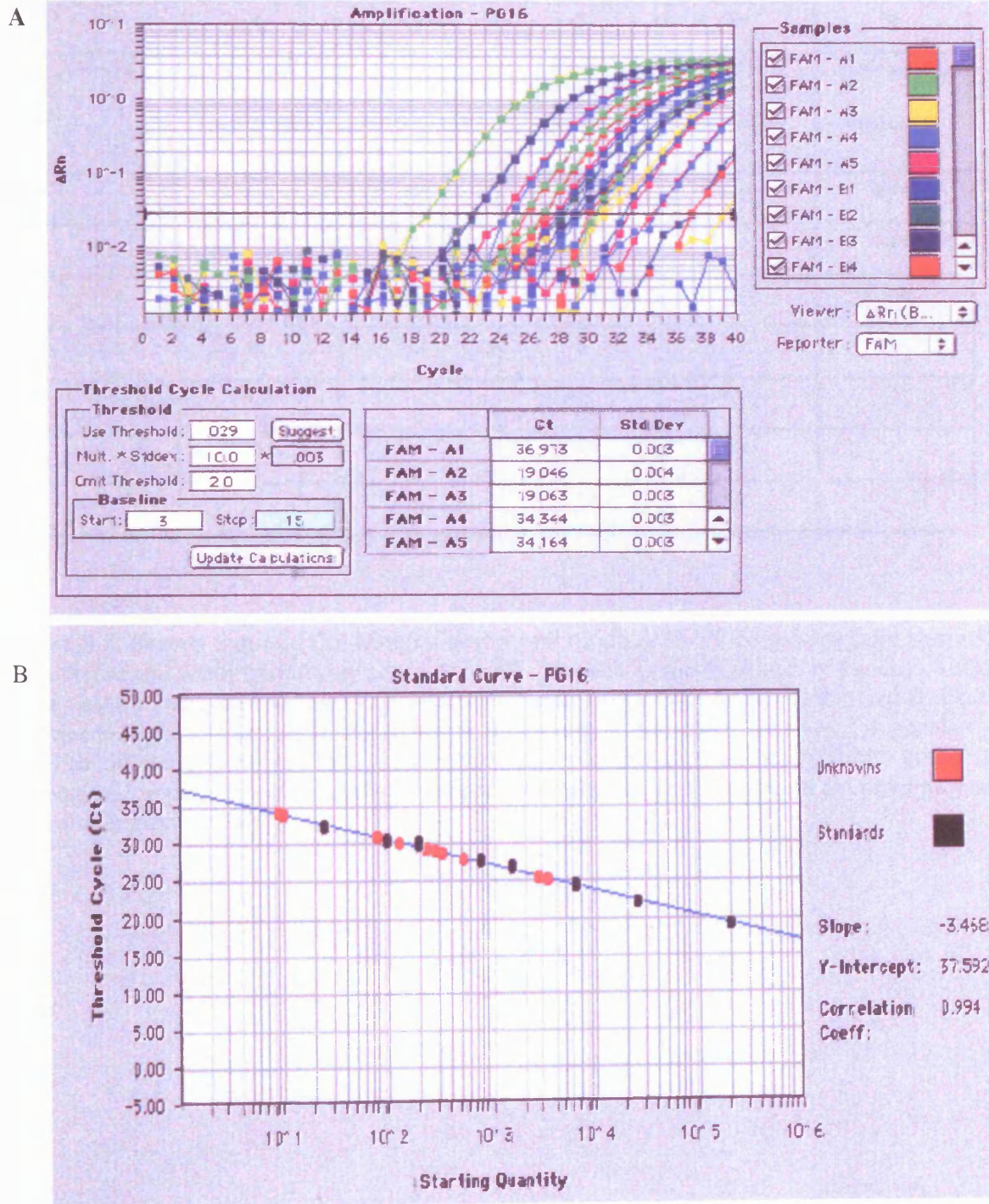


Figure 5.1. Representative data from QPCR analysis of pVAC1.NT gold slurries. The minimum number of cycles required for detection of each pVAC1.NT containing sample (Ct) is shown in A and the resulting values are plotted against a pVAC1.NT standard curve for copy number determination (B)

	A	B	C	D	E
Group name	Theoretical loading ng DNA / cartridge	pVAC1.NT in vitro study ng DNA / cartridge	pVAC1.NT primary immunisation ng DNA/ cartridge	pVAC1.NT boost immunisation ng DNA/ cartridge	Theoretical loading copies / gold bead
a	500	500	500	500	24,368
b	50	71.5	41	66	2,436
c	5	6.2	8	6	243
d	0.5	0.5	1	0.5	24
e	0.05	-	0.15	0.05	2
f	0.005	-	0.05	0.005	-
g	0	-	0	0	-

Table 5.2. Results from QPCR analysis performed on pVAC1.NT containing gold slurries. The theoretical loading quantity of pVAC1.NT for each group is shown in panel A. Gold slurry used for the first *in vitro* experiment (refer to Figure 5.2) is shown in panel B. Gold slurries used for manufacture of cartridges for *in vivo* experiments are shown in panels C-D. The theoretical number of DNA copies/gold particle for each pVAC1.NT group is represented in panel E. DNA copies/gold particle calculations are based on the precipitation of a single plasmid only.

5.3. Results

For all *in vitro* and *in vivo* dose response experiments, the nomenclature used to denote amount of NT encoding DNA delivered by PMDD is shown in Table 5.1. To facilitate comparison of data, the *in vitro* results are expressed as number and area of IFN- γ producing cells/million responder cells and the *in vivo* results are expressed as number of IFN- γ producing cells/million responder cells.

5.3.1. Effect of immunising DNA dose on CD8+ T cell responses *in vitro* and *in vivo*

5.3.1.1. QPCR analysis

Plasmid pVAC1.NT (in log dilutions from 500–0 ng) was mixed with different amounts of pVAC1 plasmid (Table 5.1). The total amount of DNA/cartridge was 500 ng. Prior to the *in vitro* and *in vivo* dose response studies, QPCR analysis was carried out on gold slurries from a certain number of DNA cartridge batches to confirm the correct ratio of pVAC1.NT against pVAC1. The analyses involved calculating the number of amplification cycles required for measurement of specific plasmid copy number in each sample (known as cycle threshold or Ct) and comparing these values against a standard curve of known copy numbers (Figure 5.1). For the *in vitro* study, results presented in Table 5.2, column B represent the QPCR analysis of the cartridges used in the first dose response study. For the first *in vivo* study, cartridges prepared for the boost (column D) compared more favourably with the theoretical DNA loading (column A) than did cartridges prepared for the primary immunisation (column C). Overall, QPCR analyses verified that the pVAC1.NT plasmids precipitated in the expected proportion with approximately a log difference between successive groups in cartridge batches. This difference is maintained even at the lowest concentration of plasmid (i.e. 0.005 ng). The theoretical number of pVAC1.NT DNA copies/gold bead is shown in column E. The columns shows that on average, cartridges containing 0.5 ng DNA result in 24 copies of NT plasmid been precipitated on individual gold beads, while 0.05 ng gives 2 copies of plasmid/gold bead. These calculations are based

on a DNA loading rate of 2 $\mu\text{g}/\text{mg}$ gold, that 1 mg gold contains 1.22×10^7 gold particles and that 1 copy of pVAC1.NT plasmid contains 4.05×10^6 pg DNA/pmole.

5.3.1.2. CD8+ restricted T cell responses to pVAC1.NT *in vitro*

The pVAC1.NT/pVAC1 dose responses were investigated *in vitro* using the CD8+ T cell assay (i.e. MC57s transfected with pVAC1.NT were used as APCs to activated RAG1.F5 splenocytes). The *in vitro* functional assay conditions and IFN- γ detection are described in sections 2.8.3 and 2.9, respectively. The two batches of cartridges used in parallel *in vivo* experiments were assessed *in vitro*. Each batch contained a dose response of pVAC1.NT against pVAC1 (500–0 ng). Detection was reported by number and area of IFN- γ producing cells/million cells. Results of the first batch are shown in Figure 5.2, row A and relate to *in vivo* experiments represented in Figures 5.4A and 5.5A. Significance was obtained in the 500 ng group only when the data was reported by number and area of IFN- γ producing cells/million cells.

Similar analyses were performed for the second batch of cartridges (*in vivo* data represented in Figures 5.4B and 5.5B) and are shown in Figure 5.2, row B. Results reveal that only the 500 ng group was significantly above control when data was reported by area of IFN- γ producing cells/million cells while the 500 and 50 ng groups achieved significance when data was reported by number of IFN- γ producing cells/million cells.

To investigate the relationship between number and area of data reporting further, both methods were compared for the pVAC1.NT/pVAC1 dose response studies (Figure 5.3, panels A and B). R^2 values of 0.993 and 0.984 were obtained for the first and second batch of cartridges, respectively. When this type of correlation was extended to include all *in vitro* assays performed ($n=8$), a R^2 value of 0.988 was obtained (Figure 5.3, panel C). These results suggests that both methods are similar and that either one may be used to report the *in vitro* functional data.

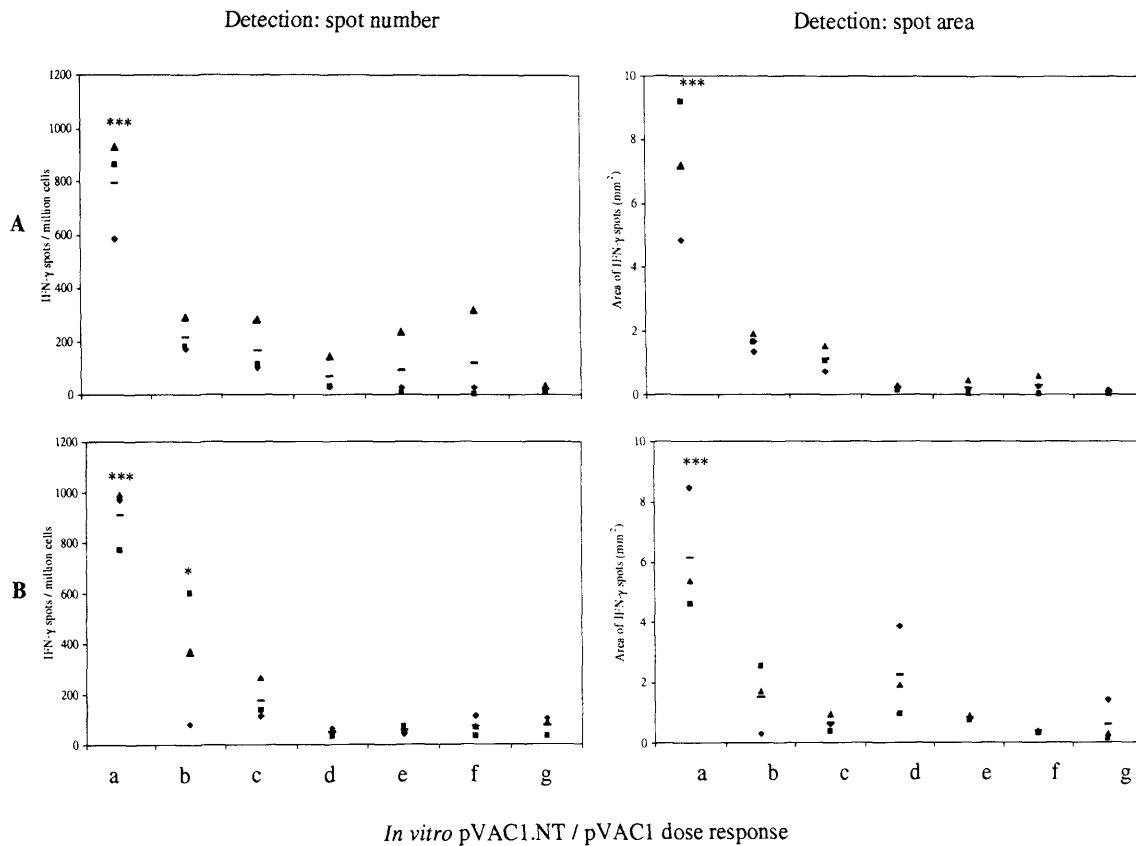


Figure 5.2. Activation of NT specific activated RAG1.F5 splenocytes by MC57 cells as measured by IFN- γ ELISPOT. MC57s were transfected with pVAC1.NT/pVAC1 containing cartridges and incubated overnight in a 1:1 ratio with 1×10^5 cultured RAG1.F5 splenocytes and 200 ng/ml IL-2. Initial and repeat experiments are shown in row A and B, respectively. Detection was reported by number (left hand column) and area (right hand column) of IFN- γ producing cells/million cells. Each symbol (except bars) corresponds to an individual experiment. Bars are the mean of all experiments for each group. For each individual experiment, data is mean of triplicate analyses of pooled triplicate transfections. Stars on top of groups represent Dunnet's test comparison of group means with corresponding control groups (group g) (* $p < 0.05$, *** $p < 0.001$).

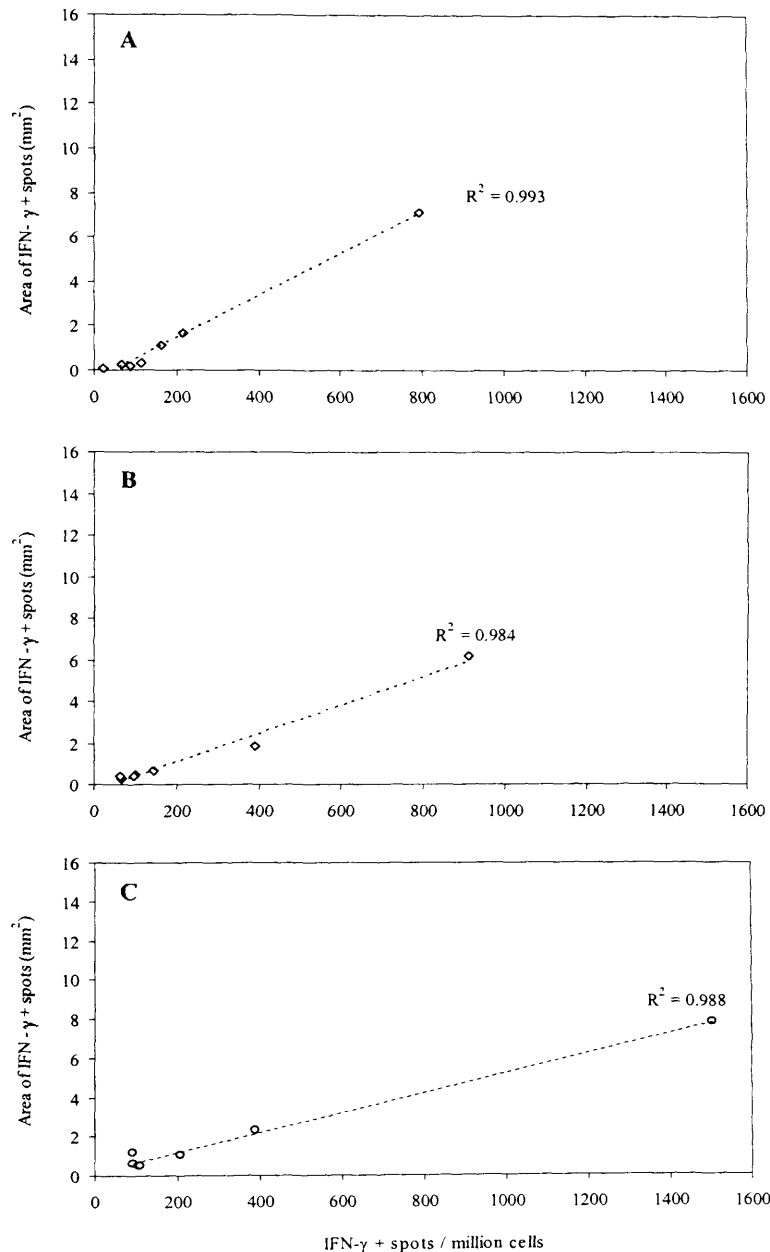


Figure 5.3. Comparison of data reporting methods for the pVAC1.NT/pVAC1 dose response studies. Panels A and B represent correlation plots of dose response studies shown in Figure 5.2, row A and row B, respectively. Panel C: Correlation plot of all *in vitro* pVAC1.NT/pVAC1 dose response studies performed (n=8). Mean values of number (x-axis) and area (y-axis) of IFN-γ producing cells/million cells from each group (denoted by horizontal bars in Figures 5.2) were used for correlation and linear trend-line regression coefficient (R^2) was fitted to data.

5.3.1.3 CD8+ restricted T cell responses to pVAC1.NT *in vivo*

The pVAC1.NT/pVAC1 plasmid containing batches were also assessed in two *in vivo* dose response studies. CD8+ specific T cell responses were measured by IFN- γ ELISPOT and intracellular IFN- γ staining (see section 2.13). For the ELISPOT data, results presented are the number of IFN- γ spots in the media + IL-2 + peptide group minus the media + IL-2 group.

In the IFN- γ ELISPOT analysis of the first experiment (Figure 5.4A), significant results were obtained in the 500 ng pVAC1.NT group only at day 14 post primary (PP). After boost, the sensitivity and magnitude of the pVAC1.NT response increased. Significance was obtained in groups down to 5 and 0.05 ng pVAC1.NT at days 7 and 14, respectively. Responses above control were observed in the 500, 50 and 0.5 ng groups at day 7 post boost (PB) but these groups were not statistically significant due to the high background levels of IFN- γ associated with these groups. In the repeat experiment (Figure 5.4B), significant results were obtained at each time-point. Groups immunised with 500, 50 and 5 ng pVAC1.NT were significantly above control at day 7 PP while the 500 and 50 ng groups achieved significance at day 14 PP. Maximum sensitivity and magnitude of the pVAC1.NT response was observed at day 7 PB where significance was obtained in groups down to 0.5 ng. This response decreased at day 14 PB where groups down to 5 ng were significantly above control. The magnitude of the response also decreased at this time-point. For example, the frequency of cytokine producing cells in the 50 ng group at day 14 PB was ~450 spots/million cells compared with ~200, ~400 and ~1100 at days 7 and 14 PP and day 7 PB, respectively. The lowest amount of pVAC1.NT capable of eliciting a response above control as detected in both experiments was 0.05 and 0.5 ng in the first and second experiments, respectively.

The intracellular IFN- γ staining analysis of the first dose response study is shown in Figure 5.5A. Groups immunised with 500 and 50 ng were significantly different from the control group at day 14 PP. After boost, the pVAC1.NT response increased with significance reached in groups down to 0.5 ng at day 7 and 14. The magnitude of the response was

greatest at day 7 PB (e.g. in the 50 ng group, the frequency of CD8_{high} CD62L_{low} IFN- γ producing cells was 1.9 % at this time-point compared with 0.25 and 0.78 % at day 14 PP and day 14 PB, respectively). In the second experiment (Figure 5.5B), significant results were obtained in groups down to 5 ng pVAC1.NT at all time-points. The sensitivity of the pVAC1.NT response increased after boost with significance reached in the 0.5 ng group at day 7 PB. The maximum response was obtained at day 14 PB (e.g. the frequency of CD8_{high} CD62L_{low} IFN- γ producing cells in the 50 ng group was 0.6 % at this time-point compared with 0.29, 0.57 and 0.31 % at day 7 and 14 PP and day 7 PB, respectively). Representative flow cytometry analysis of the 500 ng pVAC1.NT and pVAC1 groups at day 14 PB is shown in Figure 5.6. In conclusion, the lowest amount of pVAC1.NT capable of eliciting a response above control as detected in both experiments was 0.5 ng.

5.3.1.4. Comparing the *in vitro* model with CD8+ T cell responses *in vivo*

To investigate the relationship between the *in vitro* functional assay and CD8+ responses *in vivo*, the *in vitro* pVAC1.NT/pVAC1 dose response data was plotted against the *in vivo* IFN- γ ELISPOT and intracellular IFN- γ staining dose response data. Analysis was performed on two batches of cartridges which were examined both in the *in vivo* and *in vitro* assays. For the *in vitro* pVAC1.NT/pVAC1 dose responses, data generated using number of IFN- γ producing cells/million cells as the reporting method was used for correlation.

The regression coefficients obtained from both sets of plots for the second batch of cartridges are shown in Figure 5.7. When the *in vitro* data (represented in Figure 5.2, row B) was plotted against the *in vivo* IFN- γ ELISPOT data (represented in Figure 5.4B), results revealed a good correlation between both sets of data points (Figure 5.7, left hand column). The best correlation was achieved at day 7 PP (i.e. a R^2 value of 0.997). This value decreased to 0.822 and 0.648 at day 14 PP and 7 PB, respectively before increasing to 0.830 at day 14 PB. This trend was not observed when the *in vitro* data was plotted against the *in vivo* intracellular IFN- γ staining (represented in Figure 5.5B). The optimal correlation

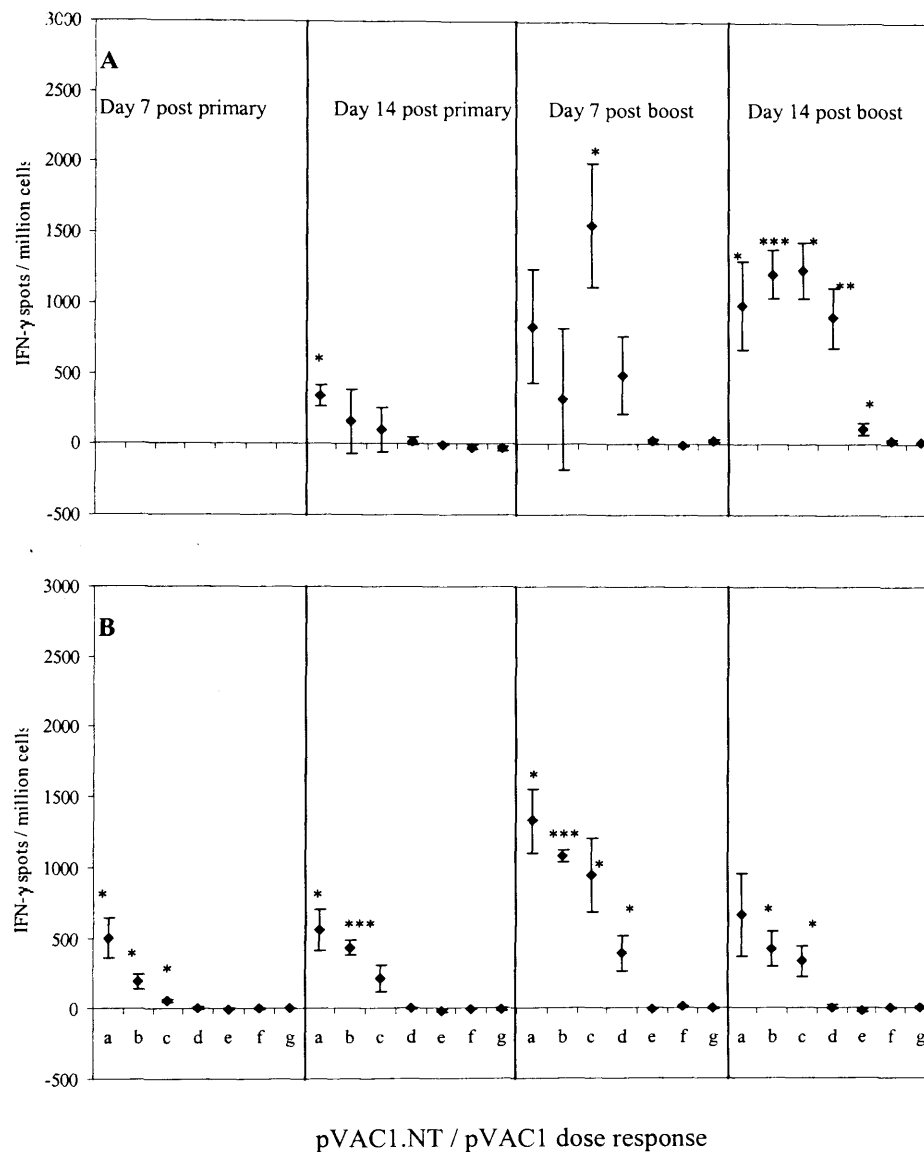


Figure 5.4. NT specific IFN- γ producing CD8⁺ T cells detected by ELISPOT. Mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1 dose response and spleens collected at day 14 PP and days 7 and 14 PB in the initial experiment (A) and at days 7 and 14 PP and PB in the repeat (B). Prior to ELISPOT analysis, splenocytes were cultured overnight in the presence of media + 50 ng/ml IL-2 or media + 50 ng/ml IL-2 + 100nM NT peptide. Results are mean of individual means \pm SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with pVAC1 only group (group g) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

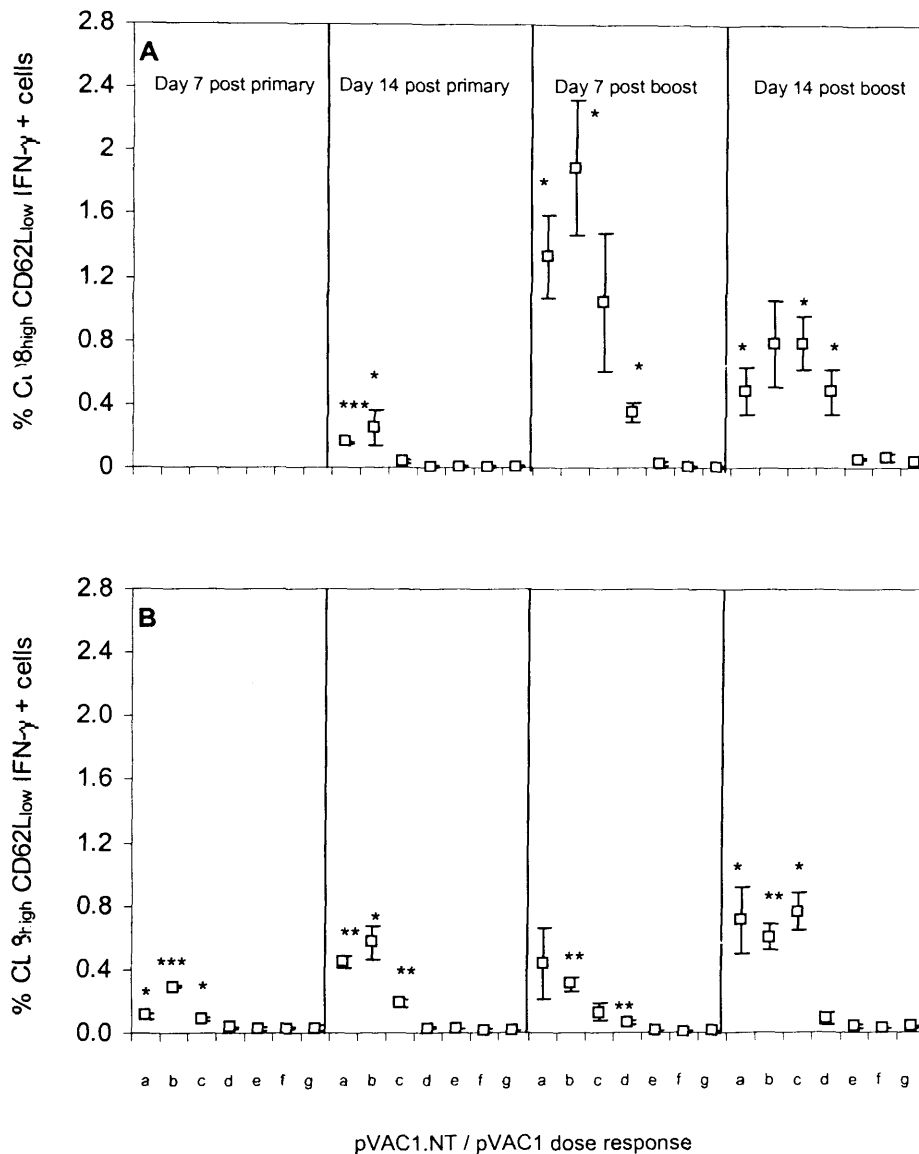


Figure 5.5. NT specific CD8⁺ T cell responses measured by IFN- γ intracellular staining. Mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1 dose response and CD8 T cell responses detected as NT specific IFN- γ producing cells in splenocytes collected at day 14 PP and days 7 and 14 PB in the initial experiment (A) and days 7 and 14 PP and PB in the repeat experiment (B). Splenocytes were incubated with 100 nM NT for 6 hrs and 10 $\mu\text{g/ml}$ Brefeldin A (last 4 hrs) before staining and flow cytometry analysis. Results are mean of individual means \pm SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with pVAC1 only group (group g) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

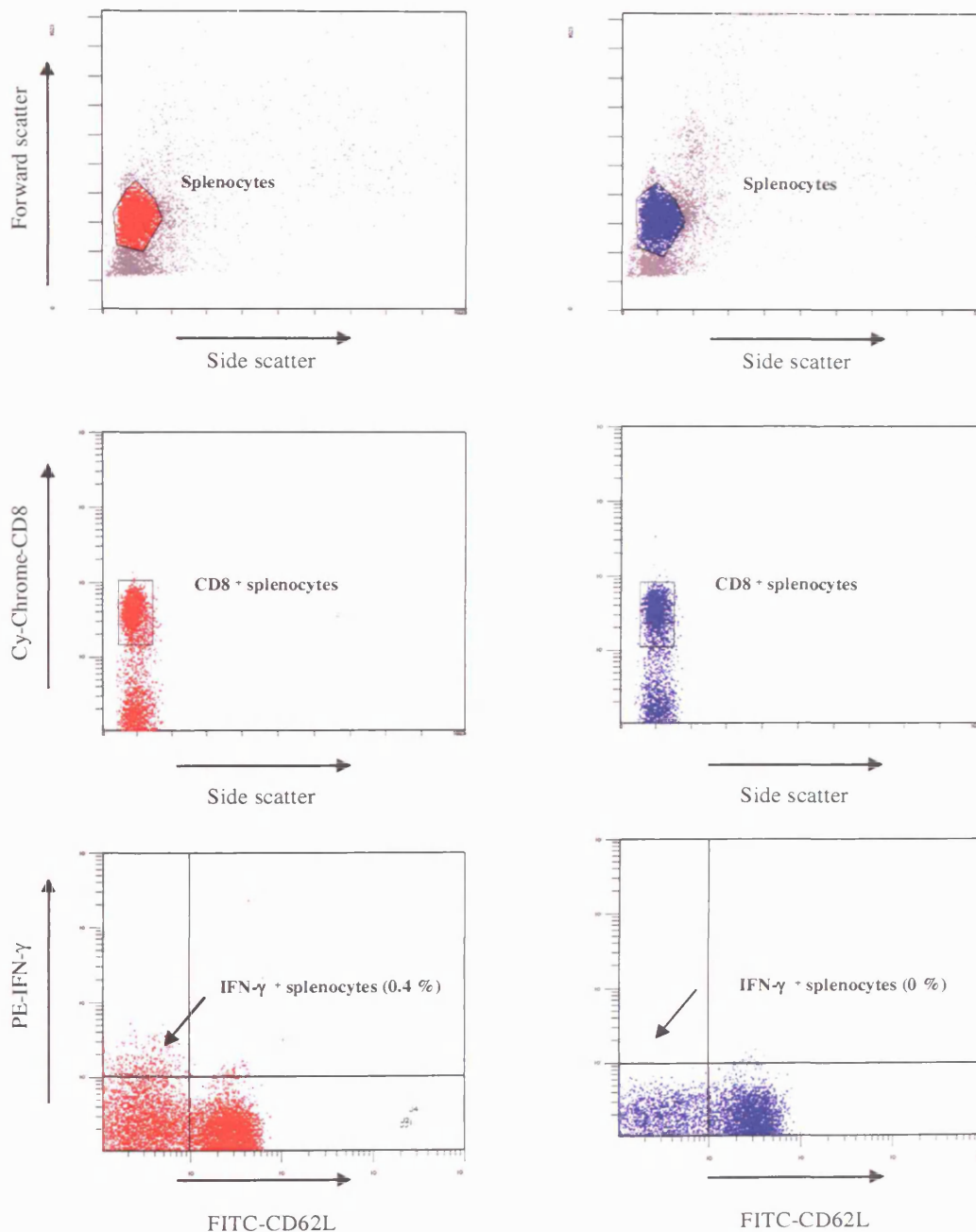
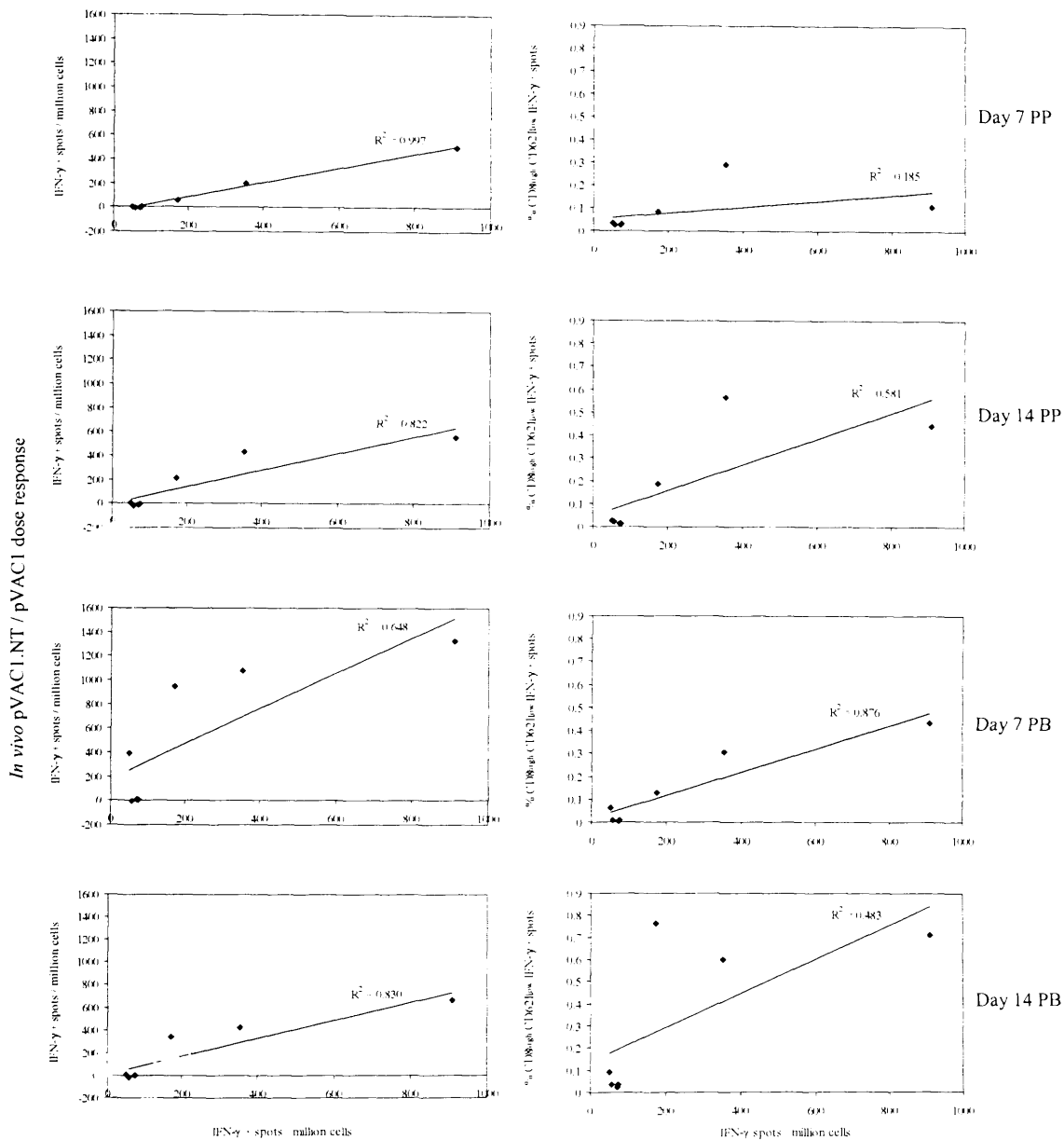


Figure 5.6. Representative data from flow cytometry analysis of NT specific IFN- γ producing CD8⁺ T cells. Data is from the day 14 PB pVAC1.NT/pVAC1 dose response (shown in Figure 5.5A) and compares the intracellular IFN- γ production in spleen cells of mouse immunised with 500 ng pVAC1.NT (left hand column) with mouse immunised with control group (right hand column). 5×10^5 splenocytes were collected for each analysis and CD62L_{low} and IFN- γ + cells (final row) were gated on the CD8_{high} population (middle row).



In vitro pVAC1.NT / pVAC1 dose response

Figure 5.7. Comparison of the *in vitro* and *in vivo* pVAC1.NT/pVAC1 dose response studies for the second batch of cartridges. The *in vitro* IFN- γ ELISPOT data (Figure 5.2B) was plotted against the *in vivo* IFN- γ ELISPOT (Figure 5.4B) and intracellular IFN- γ staining data (Figures 5.5B) and the resulting correlation plots are shown on the left hand and right hand columns, respectively. The dose responses were compared for the day 7 PP, day 14 PP, day 7 PB and day 14 PB *in vivo* time-points. Mean values was used for correlation and linear trend-line regression coefficient (R^2) was fitted to data.

R ² values from the <i>in vitro</i> IFN- γ ELISPOT / <i>in vivo</i> IFN- γ ELISPOT correlation plots		
Time-point	Cartridge batch 1	Cartridge batch 2
Day 7 PP	-	0.997
Day 14 PP	0.885	0.822
Day 7 PB	0.144	0.648
Day 14 PB	0.180	0.830

Table 5.3. Comparison of the *in vitro* and *in vivo* pVAC1.NT/pVAC1 dose response studies for the first and second batch of cartridges. R² values were obtained by plotting *in vitro* IFN- γ ELISPOT data against the *in vivo* IFN- γ ELISPOT data.

from this analysis was 0.876 obtained at day 7 PB (Figure 5.7, right hand column). The regression coefficients from the *in vitro* IFN- γ ELISPOT/*in vivo* IFN- γ ELISPOT correlation plots for the first and second batch of cartridges are represented in Table 5.3. For both sets of data, the best correlations were achieved when the *in vitro* data was plotted against the data from the early *in vivo* time-points (i.e. R^2 values of 0.855 and 0.997 for day 7 PP using cartridge batch 1 and day 14 PP using cartridge batch 2, respectively).

5.3.2. Effect of a promoter dose response on CD8+ NT response *in vitro* and *in vivo*

5.3.2.1. Cloning of p7313.NT plasmids

Four p7313.NT plasmids with different promoter configurations were constructed as described in chapter 2 (Figure 5.8). Firstly, p7313.plc.NT was synthesised by the ligation of the NT insert with the p7313.plc vector. Following this, plasmids p7313.ii.NT, p7313.me.NT and p7313.m.NT were constructed. After transformation and overnight incubation on agar plates, selected colonies were scaled up and the purified plasmid analysed for correct vector sequence by Bam H1 and Cla1 restriction enzyme digest. All vectors showed correct DNA fragment profile (Figure 5.9).

5.3.2.2. Characterisation of p7313.NT plasmids

Each of the p7313 plasmids were transfected into MC57 cells by PMDD on three separate occasions and antigen production monitored by Western blot analysis of cell lysates and by the *in vitro* CD8+ T cell assay. A representative Western blot analysis is shown in Figure 5.10A. The plasmid containing the weakest promoter (p7313.m.NT) was undetectable suggesting that this promoter configuration (minimal CMV promoter only) is extremely inefficient at driving RNA transcription relative to the other promoters. The ii.NT plasmid appears to be the strongest p7313 plasmid generating more NT antigen than the corresponding pVAC1.NT plasmid. The *in vitro* response measured by IFN- γ ELISPOT

broadly correlated to expression level (Figure 5.10B). However, only the ii.NT plasmid was significantly greater than the control plasmid (i.e. pCMV-GFP).

5.3.2.3. NT promoter dose response *in vitro*

Cartridges containing the 50 ng p7313 plasmids were compared against pVAC1.NT/pVAC1 dose responses *in vitro*. The results for the first set of cartridges are represented in Figure 5.11. Results show that the ii and plc.NT groups were clearly the strongest with these groups been significantly different from the empty p7313 plasmid control. Overall, the *in vitro* data presented in Figures 5.10 and 5.11 suggest that the ii and plc.NT are the best p7313 plasmids while the me.NT and especially m.NT are weaker.

5.3.2.4. The NT promoter dose response *in vivo*

The strength of the p7313 plasmids was also examined *in vivo*. In the first *in vivo* experiment, cartridges containing 50 ng of the different p7313 plasmids were compared against a pVAC1.NT/pVAC1 dose response. CD8+ specific T cell responses were monitored using IFN- γ ELISPOT and IFN- γ intracellular staining at day 14 PP and days 7 and 14 PB. For the repeat experiment, cartridges containing 5 ng p7313 plasmids were compared against a pVAC1.NT/pVAC1 dose response with responses monitored at day 14 PP and day 7 PB. For the IFN- γ ELISPOT analysis, results presented are the number of IFN- γ spots in the media + IL-2 + peptide group minus the media + IL-2 group. Results from the first *in vivo* study revealed that the ii, plc and me.NT plasmids were significantly greater than the empty p7313 plasmid at all time-points (Figure 5.12A). Following boost, the magnitude of the IFN- γ response increased to a maximum level at day 7 with the me.NT plasmid yielding the strongest response (~1250 spots/million cells). In the second experiment, a similar trend emerged. The ii, plc and me.NT plasmids were significantly greater than the empty p7313 plasmid at the day 7 PB time-point (Figure 5.12B). However, unlike the first analysis, the ii.NT proved to be the best p7313 plasmid yielding the greatest response at days 14 PP and 7 PB. At day 7 PP, the ii and plc.NT groups were comparable to

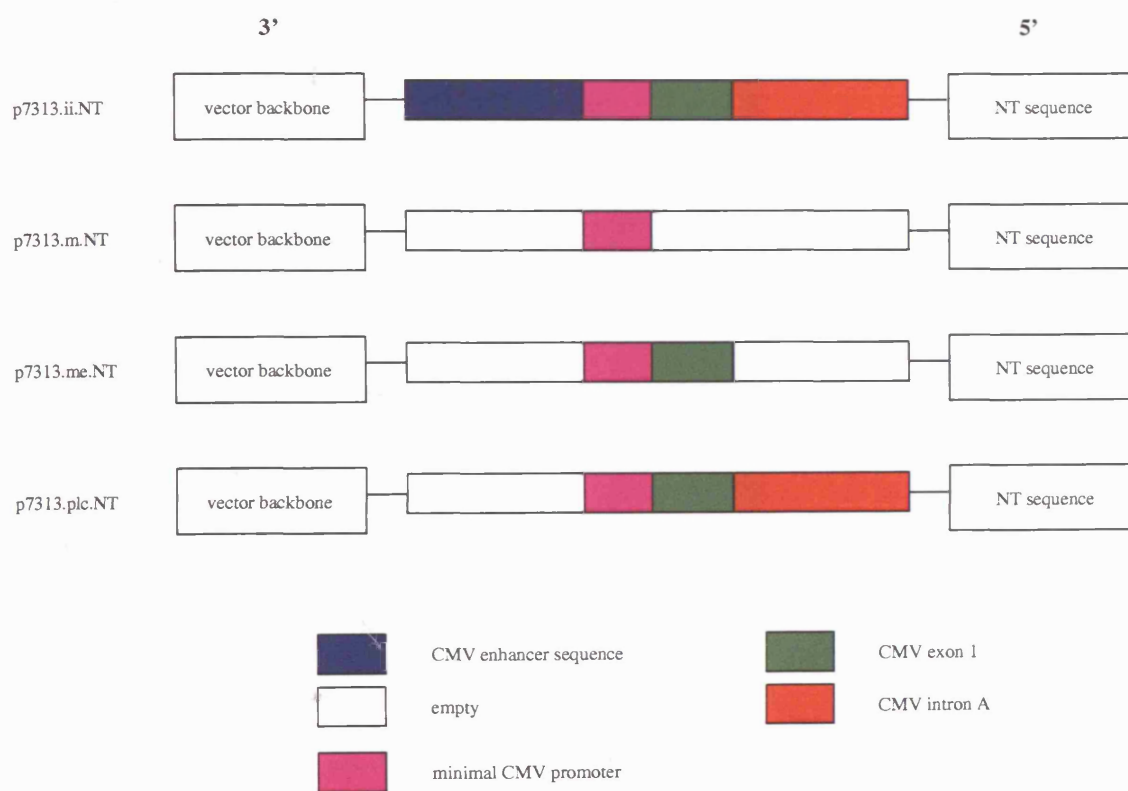
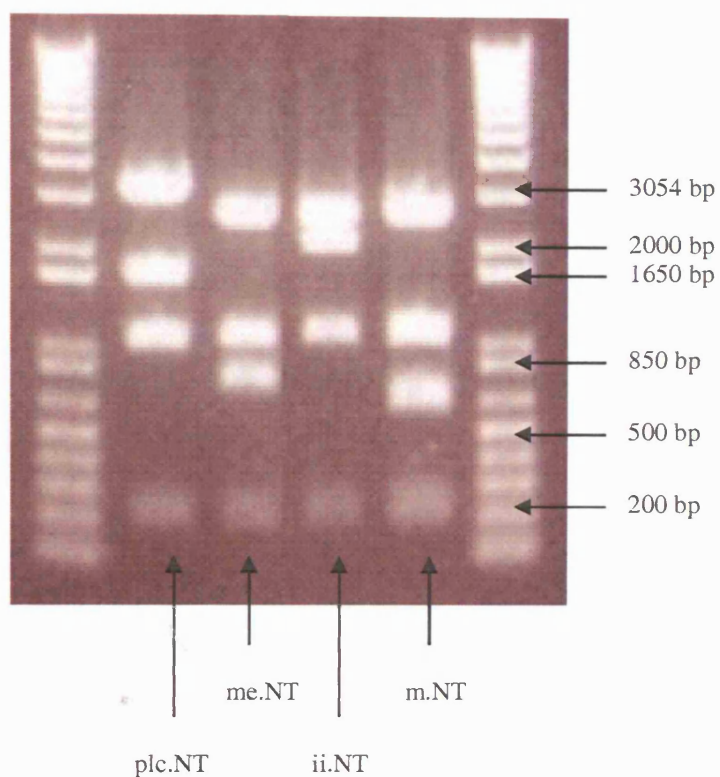


Figure 5.8. Schematic of the different p7313.NT promoter configurations used.



DNA fragment size after Cla I and Bam HI digest				
plc.NT	216	1038	1614	3067
me.NT	216	770	1050	2493
ii.NT	216	1050	1982	2493
m.NT	216	651	1050	2493

Figure 5.9. Bam HI and ClaI restriction enzyme digest of the p7313 plasmids. All plasmids show correct DNA fragment profile as outlined in the table.

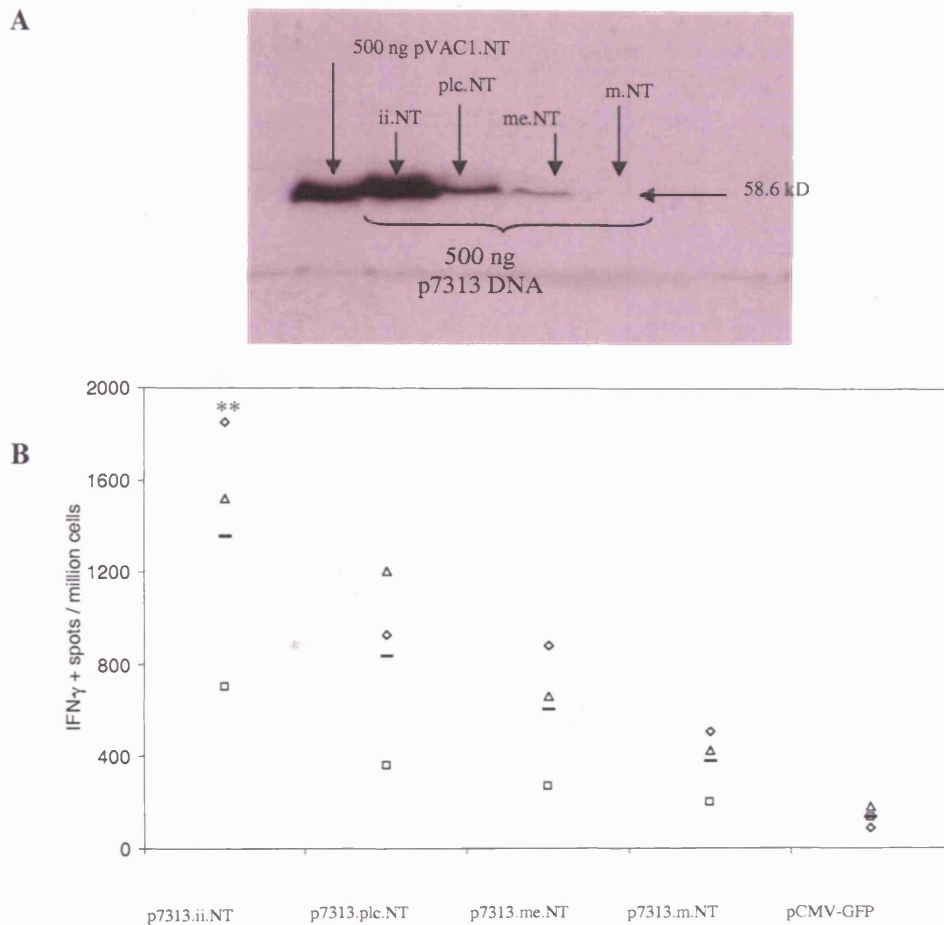


Figure 5.10. Characterisation of p7313.NT plasmids by Western blot analysis (panel A) and using the *in vitro* CD8⁺ T cell assay (panel B). For Western blot analysis, MC57 cells were transfected with 500 ng of p7313.NT plasmids, incubated overnight, lysed and assayed as described in section 2.12. For the *in vitro* CD8⁺ T cell assay, transfected cells were incubated overnight in a 1:1 ratio with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2 and measured by IFN- γ ELISPOT. Negative control was MC57s transfected with pCMV-GFP plasmid. Each symbol (except bars) represents an individual experiment. Bars are the mean of all experiments for each group. Stars on top of p7313.ii.NT group represent Dunnet's test comparison of group mean with negative control group mean (** $p < 0.01$).

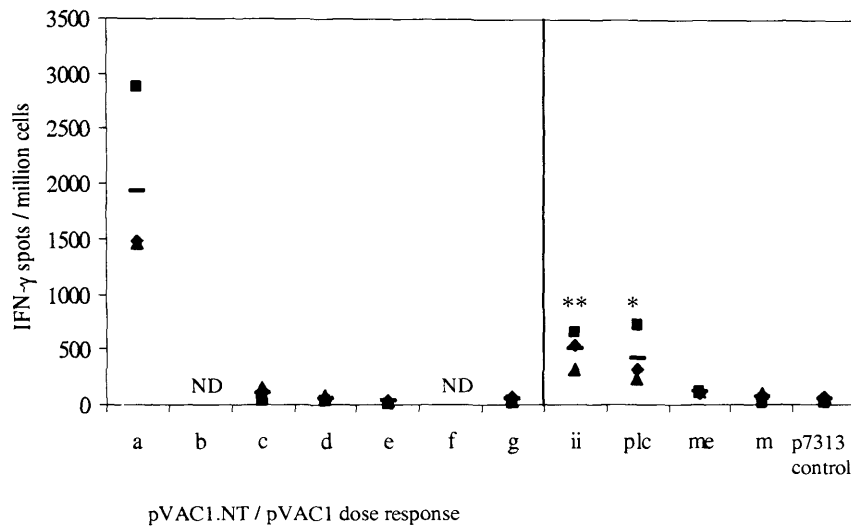


Figure 5.11. Comparison of pVAC1.NT and p7313.NT plasmid potency using the *in vitro* CD8+ T cell assay. MC57 cells were transfected with a pVAC1.NT/pVAC1 dose response and 50 ng of p7313.NT plasmids. Data is for the first batch of cartridges. Cells were incubated overnight in a 1:1 ratio with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2 and then measured by IFN- γ ELISPOT. Each symbol (except bars) represents an individual experiment. Bars are the mean of all experiments for each group. Stars on top of the ii.NT and plc.NT groups represent Dunnet's test comparison of group means with the p7313 control group mean (* $p < 0.05$, ** $p < 0.01$). ND: not done.

the corresponding 5 ng pVAC1.NT group (i.e. group C), but after boost, only the ii.NT yielded a response similar to the pVAC1.NT group. For the first set of cartridges, the results obtained for intracellular IFN- γ staining are shown in Figure 5.13A. No responses were observed in any group at day 14 PP. After boost, significant responses were obtained in the ii, plc and me.NT groups at day 7 and in the ii and plc.NT groups at day 14. The data was similar to that observed in the IFN- γ ELISPOT analysis in that the me.NT plasmid was the strongest p7313 plasmid at day 7 and day 14 PB (e.g. the frequency of CD8_{high} CD62L_{low} IFN- γ producing cells was ~0.75 % at day 7 PB). The results obtained for intracellular IFN- γ staining in the repeat experiment are shown in Figure 5.13B. At day 14 PP, of the p7313 plasmids, only the group immunised with the ii.NT plasmid yielded a response above control (0.46 % of CD8_{high} splenocytes were CD62L_{low} IFN- γ producing cells). After boost, the magnitude and sensitivity of the response increased with the 5 ng p7313.ii, plc, me.NT and pVAC1.NT plasmid groups giving similar responses. Representative flow cytometry analysis of the four p7313.NT plasmids at day 7 PB in the first study is shown in Figure 5.14.

5.3.2.5. Comparing the *in vitro* model with CD8+ T cell responses *in vivo*

For the first batch of cartridges, the results from the *in vitro* IFN- γ ELISPOT assays (represented in Figure 5.11) were plotted against the corresponding *in vivo* IFN- γ ELISPOT (represented in Figure 5.12A) and IFN- γ intracellular staining (represented in Figure 5.13A) results and are presented in Figure 5.15. When the data from the *in vitro* study was compared with the *in vivo* IFN- γ ELISPOT data, the best correlation was obtained at day 14 PP (R^2 value of 0.883). After boost, the correlation coefficients decreased to 0.162 and 0.02 at days 7 and 14, respectively. The *in vivo* intracellular staining data did not correlate with the *in vitro* ELISPOT data at any time-point. Correlation coefficients of 0.364, 0.415 and 0.211 were achieved at day 14 PP, 7 PB and 14 PB, respectively.

The regression coefficients from the *in vitro* IFN- γ ELISPOT/*in vivo* IFN- γ ELISPOT correlation plots for the first and second batch of cartridges are represented in Table 5.4.

The best correlations were achieved when the *in vitro* data was plotted against the data from the post primary *in vivo* time-points (i.e. at day 14 PP, R^2 values of 0.883 and 0.835 were obtained for cartridge batches 1 and 2, respectively).

5.4. Discussion

5.4.1. Effect of immunising dose on immune response

5.4.1.1. Difference in sensitivity between the *in vitro* and *in vivo* assays

An important observation from these analyses was the difference in sensitivity (i.e. detection of the lowest statistically relevant group) between the *in vitro* and *in vivo* dose responses. Significance was generally obtained in the 500 ng DNA group only in the *in vitro* dose response studies, although the second batch of cartridges did register a dose response end-point of 50 ng when area of cytokine producing cells was used as reporting method. Although the regression coefficients obtained from the correlation plots of number versus area of cytokine producing cells suggested that both reporting methods were similar, this relationship is not absolute.

In contrast, the *in vivo* dose response curves expectedly showed a far greater level of assay sensitivity, especially after boost. For these experiments, the lowest amount of pVAC1.NT capable of eliciting a significant response was generally observed to be 0.5 ng. The one exception to this was the first dose response with IFN- γ ELISPOT as detection method. Here, significance was obtained in groups down to 0.05 ng DNA (Day 14 PB data shown in Figure 5.4A). The different results obtained may be due to the differences in the ELISPOT and intracellular staining technologies as detection methods. Theoretically, both measure the influenza₃₆₆₋₃₇₄ specific IFN- γ producing CD8⁺ T cells. ELISPOT measures the total amount of cytokine secreted from functionally active cells. The image processing software calculates number and area of cytokine spots but does not discriminate between spots that are the product of one versus more than one functionally active T cell. On the other hand,

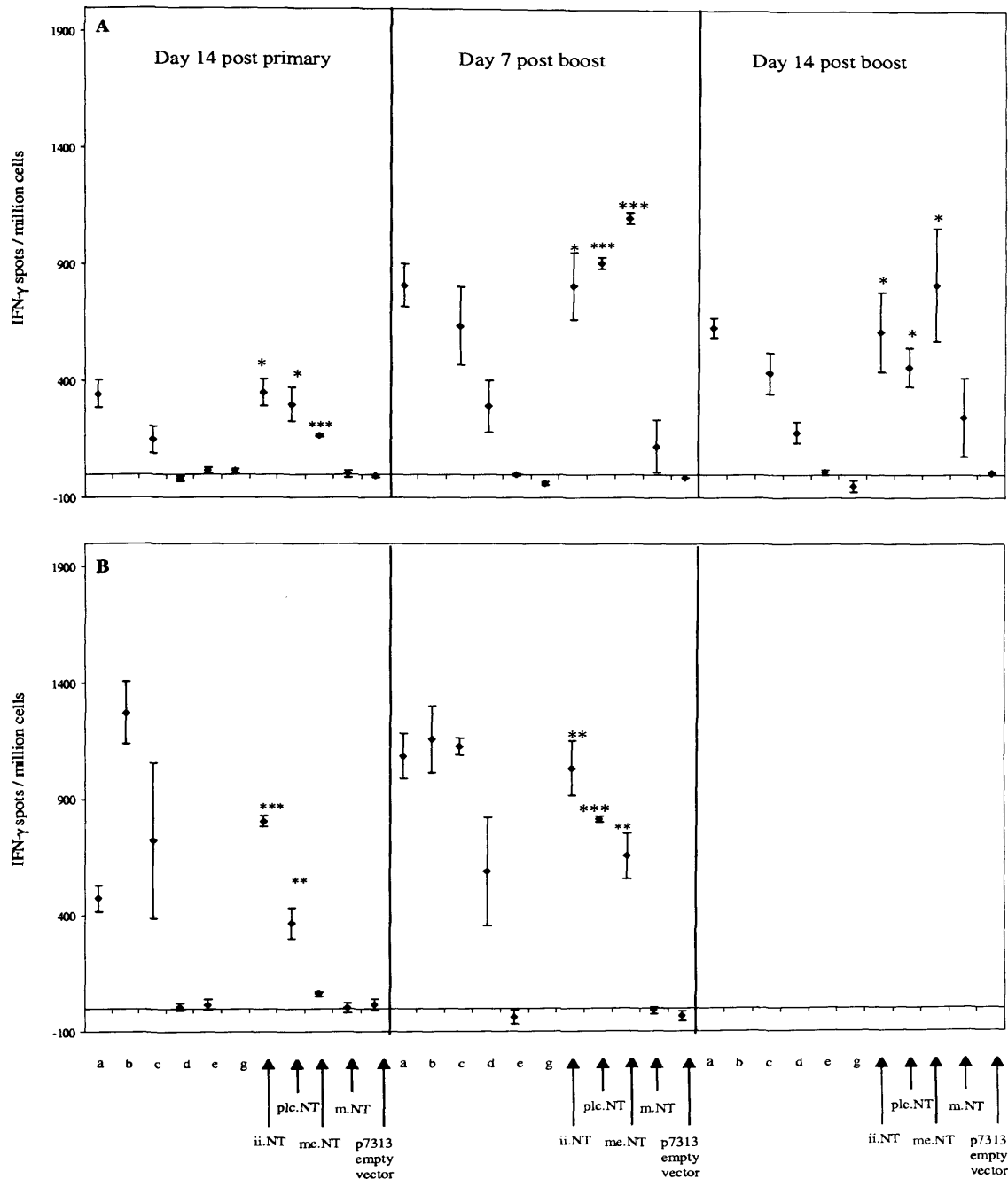


Figure 5.12. NT specific CD8+ T cell responses as measured by IFN- γ ELISPOT. For the first experiment (panel A), mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1 dose titration and 50 ng of p7313.NT plasmids and spleen cells were collected at day 14 PP and day 7 and 14 PB. For the repeat (panel B), mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1 dose response and 5 ng of p7313.NT plasmids and responses monitored at day 14 PP and day 7 PB. Results are mean of individual means \pm SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with p7313 control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

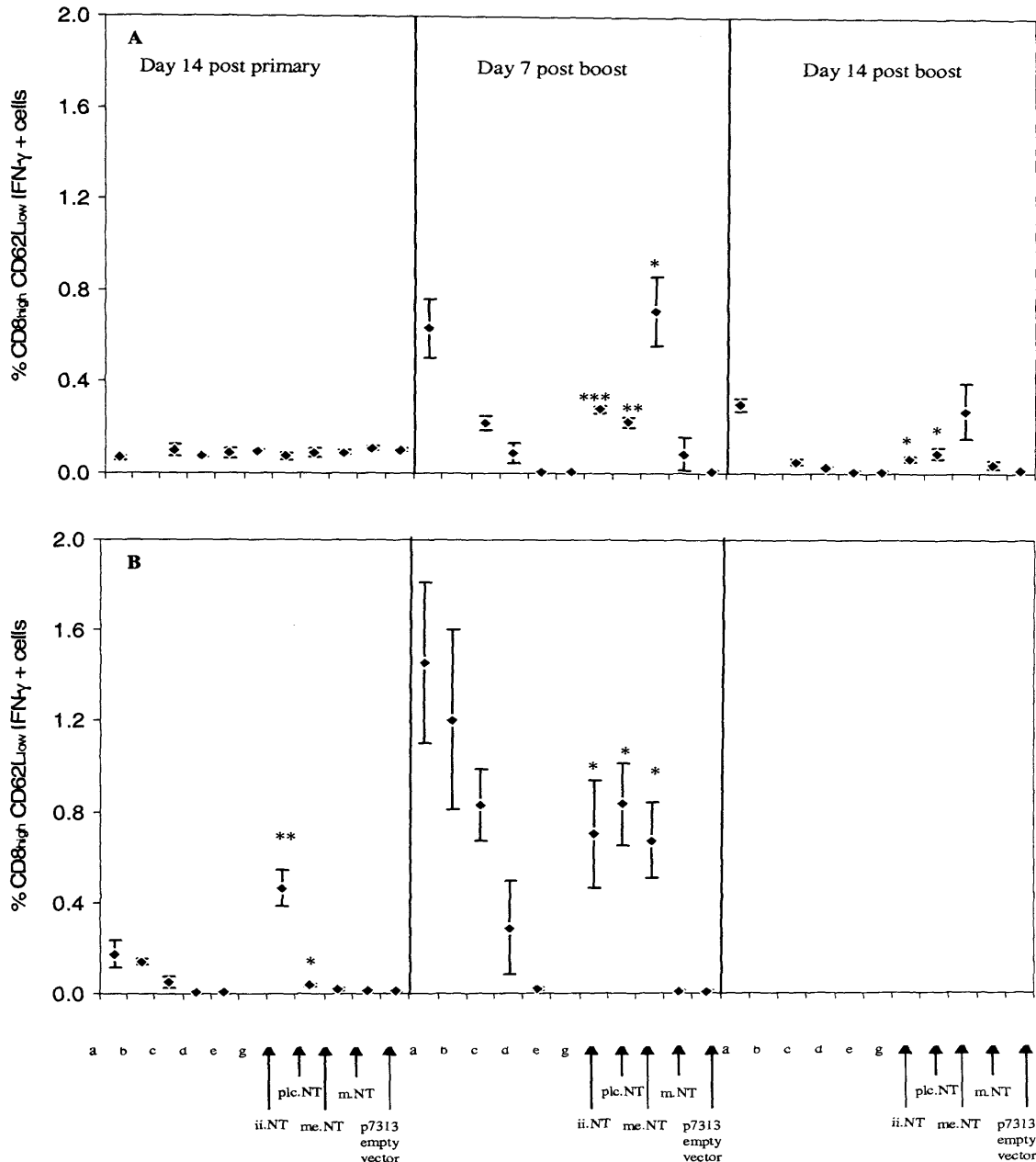


Figure 5.13. CD8⁺ T cell responses assessed by IFN-γ intracellular staining. For the first experiment (panel A), mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1 dose response and 50 ng of p7313.NT plasmids and spleen cells were collected at day 14 PP and days 7 and 14 PB. For the repeat (panel B), mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1 dose response and 5 ng of p7313.NT plasmids and spleen cells collected at day 14 PP and day 7 PB. Splenocytes were incubated in the presence of 100 nM NT for 6 hrs and 10 μg/ml Brefeldin A (last 4 hrs) before staining and flow cytometry analysis. Results are mean of individual means ± SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with p7313 control group (* p < 0.05, ** p < 0.01, *** p < 0.001).

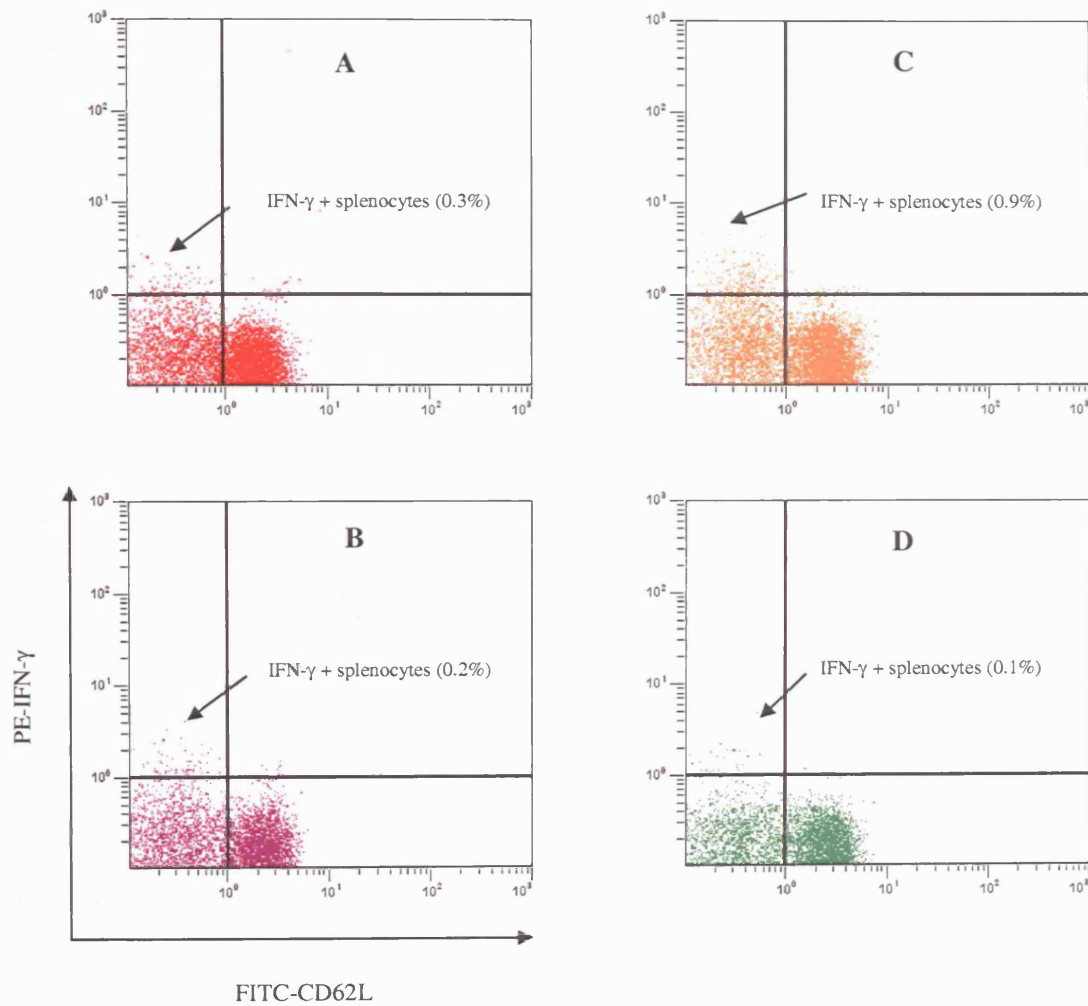


Figure 5.14. Representative data from flow cytometry analysis of NT specific IFN- γ producing CD8+ T cells. Data is taken from day 7 PB of the first experiment (described in Figure 5.13A) and compares intracellular IFN- γ production in spleen cells of mouse immunised with 50 ng of plasmids p7313.ii.NT (A), p7313.plc.NT (B), p7313.me.NT (C) and p7313.m.NT (D). 5×10^5 splenocytes were collected for each analysis and were gated on the CD8_{high} population.

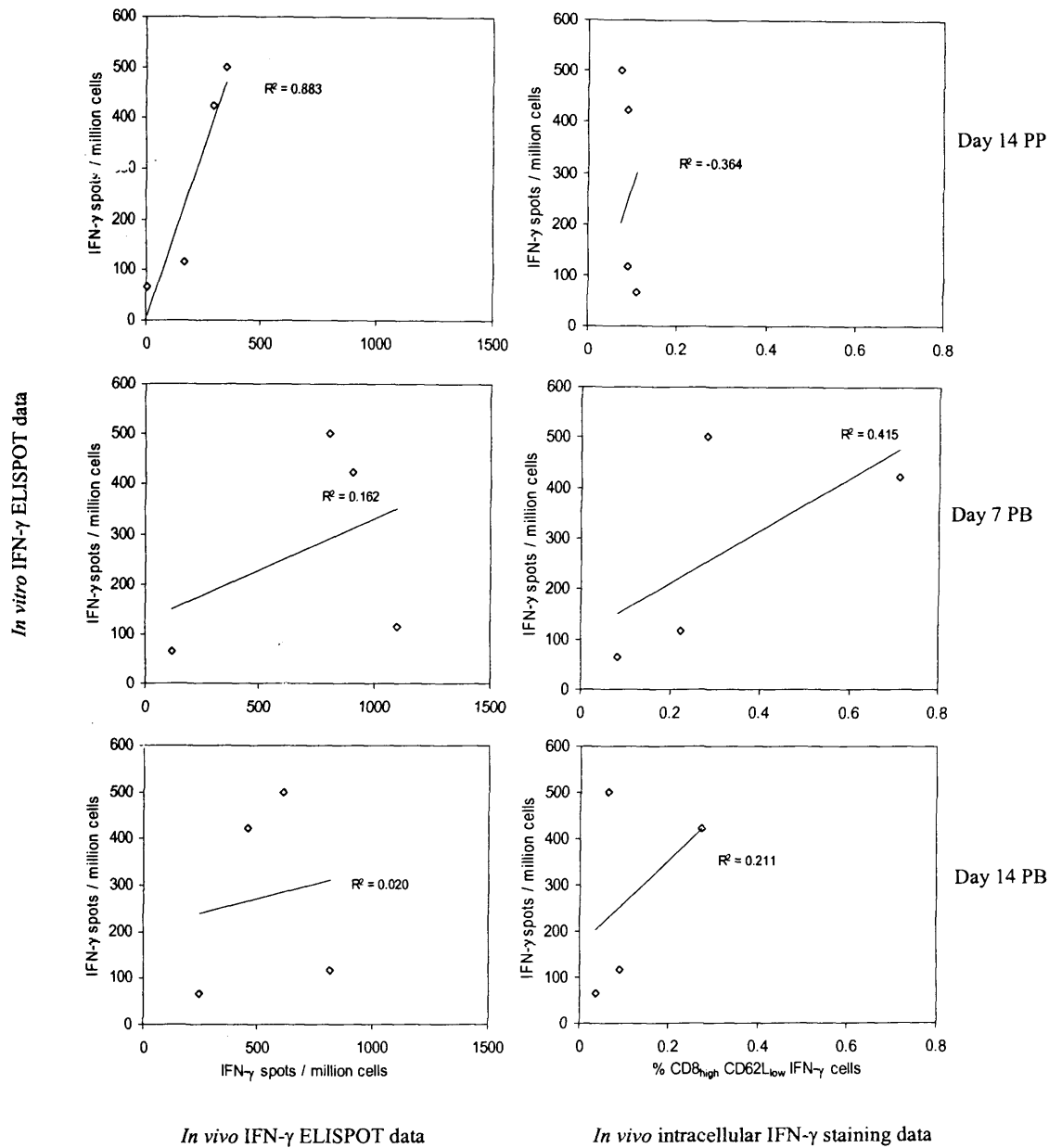


Figure 5.15. Comparison of p7313 plasmids *in vitro* and *in vivo* for the first batch of cartridges. The *in vitro* IFN- γ ELISPOT data (Figure 5.11) was plotted against the *in vivo* IFN- γ ELISPOT (Figure 5.12A) and intracellular IFN- γ staining data (Figures 5.13A) and the resulting correlation plots are shown in the left hand and right hand columns, respectively. The dose responses were compared for the day 14 PP, day 7 PB and day 14 PB *in vivo* time-points. Mean values was used for correlation and linear trend-line regression coefficient (R^2) was fitted to data.

R ² values from the <i>in vitro</i> IFN- γ ELISPOT / <i>in vivo</i> IFN- γ ELISPOT correlation plots		
Time-point	Cartridge batch 1	Cartridge batch 2
Day 14 PP	0.883	0.835
Day 7 PB	0.162	0.689
Day 14 PB	0.020	-

Table 5.4. Comparison of p7313 plasmids *in vitro* and *in vivo* for the first and second batch of cartridges. R² values were obtained by plotting *in vitro* IFN- γ ELISPOT data against the *in vivo* IFN- γ ELISPOT data.

intracellular staining measures the cytokine sequestered in the cytoplasm of individual cells and not secreted cytokine. Additionally, this technique may be compromised by sensitivity /technical issues associated with fluorescent staining of cytoplasmically localised protein. Both may offer possible explanations for the difference in results obtained for the IFN- γ ELISPOT and intracellular staining data at this time-point.

The sensitivity data obtained for the *in vivo* dose responses are in general agreement with that of Pertmer *et al.* (1995) who reported CTL responses following delivery of 16 ng DNA encoding A/PR/8/34 nucleoprotein using a similar immunisation schedule. In contradiction to results obtained here, increasing the dose above 16 ng/immunisation in that particular study did not lead to an increase in CTL lytic capacity suggesting that there was a threshold dose of DNA required for effector CTL. Once that was reached, increasing the amount of DNA administered had little effect on CTL efficacy. However, no CTL functional analysis was performed in this study so the link between CD8+ responses detected by ELISPOT and intracellular staining and CTL effector function was not established.

The sensitivity data observed in the dose responses compares well with the theoretical plasmid loading rate data presented in Table 5.2, column E. Loading 0.5 ng DNA/cartridge theoretically results in the precipitation of 24 copies of pVAC1.NT plasmid/individual gold bead while loading 0.05 ng DNA/cartridge equates to the precipitation of ~2 copies plasmid/bead. These results are in agreement with other investigators who have reported that as little as 1-20 peptide-MHC complexes can trigger activation of CTL in other systems (Sykulev *et al.*, 1996; Montoya and Del Val, 1999; Christinck *et al.*, 1991). Actually, Genequant analysis of DNA eluted from cartridges indicates that the loading rate is not the theoretical 2 μ g/mg gold but half of this. Therefore, in the case of the 0.05 ng group, this correlates to 1 plasmid copy/individual gold bead. This calculation suggests that it may be possible to elicit a response *in vivo* when gold beads carrying just a single copy of plasmid get delivered to appropriate APCs. However, trying to relate immune responses with number of plasmid copies/bead is difficult. It assumes that pVAC1.NT precipitates onto each gold bead in a homogenous manner and this may not be correct. It is not known how many gold particles penetrate the same APC and how many APC are transfected

during gene gun immunisation. The QPCR analysis which was performed on DNA gold slurries prior to cartridge manufacture confirmed that the plasmids precipitated onto gold beads in the expected proportion but it offered no information on the heterogeneity of DNA distribution/gold bead. In agreement with Creusot *et al.* (2003b), it is impossible to know whether or not the immune response raised using the DNA dose of 0.05 ng was due to the delivery to an individual APC of a single gold bead or multiple beads coated with 1 plasmid. Alternatively, an immune response may have been elicited at this dose because a bead or many beads coated with a disproportionately high number of plasmid copies (e.g. ~10 copies) may have been delivered to a single APC or multiple APCs.

5.4.1.2. Differences between primary and memory CD8+ T cell responses

During viral infection, the primary immune response is directed towards immunodominant viral epitopes resulting in selection of dominant T cell clones. In general, subsequent re-stimulation of the memory response is characterised by a quantitatively and qualitatively superior response accompanied by further epitope dependent T cell selection. In the *in vivo* pVAC1.NT dose responses, analysing the CD8+ specific responses to the immunodominant influenza₃₆₆₋₃₇₄ epitope reveals some interesting features about the development of the CD8+ T cell response. Examination of the ELISPOT and intracellular cytokine staining data shows an expected increase in the magnitude and sensitivity of the CD8+ T cell response after boosting. However, there is no indication of a linear relationship between dose administered and resulting response. After boosting, the increase is greatest in groups containing lesser quantities of pVAC1.NT (i.e. 0.05-50 ng DNA) compared with groups immunised with the top dose of pVAC1.NT and the overall difference in frequency of the influenza₃₆₆₋₃₇₄ specific CD8+ T cell subset between the top dose and lower doses becomes minimal. The data presented here compares well with others who have demonstrated that excessive levels of epitope expression ($\sim 6 \times 10^4$ epitopes/cell) only marginally increases the size of the memory response compared with that achieved at high epitope expression levels ($\sim 3 \times 10^4$ epitopes/cell) and results in a qualitatively inferior memory CTL population (Wherry *et al.*, 2002; Bullock *et al.*, 2000). Another possible explanation for the disproportionately higher increase in IFN- γ producing CD8+ T cells in groups immunised

with lower doses of pVAC1.NT compared with the top dose following boost is that excessive epitope levels may result in a skewing of the immune response from predominantly IFN- γ producing Tc1 cells to IL 10 producing Tc2 cells (Croft *et al.*, 1994; Tanchot *et al.*, 1998). While there is no direct evidence to indicate that two individual administrations of the top dose of pVAC1.NT (1000 ng) in the space of 28 days leads to the generation of excessive levels of nucleoprotein₃₆₆₋₃₇₄ epitope, this explanation does support the results obtained here. Consistent with this interpretation is the concept that T cell clones which dominate the primary response and undergo massive expansion are detectable in the memory response at lower frequencies (Callan *et al.*, 2000; Davenport *et al.*, 2002). Presumably, preferential elimination of dominant T cell clones during the primary response allows other epitope specific clonotypes to become more prevalent in the memory response thus preventing the immune response from becoming too focused. Therefore, mice immunised with increasing quantities of nucleoprotein encoding plasmid are more susceptible to immunodominant epitope T cell down regulation in the recall response. It would be interesting to monitor the response elicited against a second subdominant epitope to investigate if the T cell response to that epitope has altered with repeat exposure to antigen.

5.4.2. Effect of NT promoter dose response on immune response

The amount of NT antigen made available for presentation was varied by using NT encoding plasmids with different promoters. Construct design centred upon the ubiquitous human CMV-IE promoter which allows for constitutive expression in a wide range of cells *in vitro* and *in vivo*. Many investigators have found this promoter more effective than a range of other viral and cellular promoters (Burkholder *et al.*, 1993; Lee *et al.*, 1997; Garapin *et al.*, 2001; Zhang *et al.*, 2002). Inclusion of various molecular units such as CMV enhancer, intron A and exon 1 to this promoter resulted in a group of plasmids each capable of generating differing quantities of NT. There is much evidence which suggests that each of these promoter components contribute positively towards protein production. The CMV enhancer is a powerful and versatile unit which effectively augments CMV promoter driven transcription. From a viral perspective, it is required for the synthesis of IE gene expression

and hence the initiation of the infectious cycle (Angulo *et al.*, 1998). Its ubiquitous activity was nicely demonstrated by Schmidt *et al.* (1990) who investigated the activity of the CMV promoter-enhancer combination in transgenic mice, recording transient gene expression in twenty four of twenty eight tissues tested. However, it is noteworthy that there is also evidence that this promoter-enhancer gives limited and variable transient gene expression in studies involving a number of transgenic mouse lines (Mikkelsen *et al.*, 1992). Another positive element for CMV-IE initiated transcription is CMV-IE 1 intron A. It has been successfully used to increase gene expression in a number of transient systems and permanent cell lines *in vitro* and in transgenic mice *in vivo* (Huang *et al.*, 1990; Palmiter *et al.*, 1991). Postulated mechanisms of action include stabilisation of mRNA by splicing and/or polyadenylation (Niwa *et al.*, 1990) or by acting as an enhancer (Buchman and Berg, 1988). Its position within the transcription unit is an important consideration with placement at the 5' untranslated region (relative to CMV promoter) essential for efficient expression (Lee *et al.*, 1997). The final element incorporated into the p7313 promoter design was the CMV-IE exon-1 sequence. Although not widely recognised as important for gene expression improvement, there is evidence which demonstrates that inclusion of sequences from the initial portion of exon-1 in the presence of intron A results in a three fold increase in gene expression (Simari *et al.*, 1998).

Comparing the magnitude of the NT specific CD8+ T cell responses using different constructs in parallel *in vitro* and *in vivo* experiments provided an opportunity to examine the relationship between amount of antigen produced and resulting response following gene gun immunisation. The general observation from the *in vitro* studies was that the ii and plc.NT plasmids were the strongest p7313 plasmids and that the me and m.NT plasmids were much weaker. The second important observation was the generally broad correlation between the amount of antigen produced by these different plasmids and the resulting *in vitro* response. This suggests that the RAG1.F5 responders are responsive to the level of antigen that is processed and presented by the APCs.

A different pattern emerged *in vivo*. In the first promoter study, the me.NT plasmid gave the best response. In the second study, ii.NT was the most potent p7313 plasmid as

assessed by ELISPOT while ii, plc and me.NT gave similar results when intracellular staining was used. Although this inconsistency may be partly related to the detection method used (see section 5.4.1.1), there is still a difference between the *in vitro* and *in vivo* data. This may suggest that transient gene expression *in vitro* does not necessarily reflect the behaviour of promoters under *in vivo* conditions (Scharfmann *et al.*, 1991; Loirat *et al.*, 1999; Locher *et al.*, 2002). More specifically, there is a discrepancy between the amount of antigen produced by the different p7313 plasmids (as observed in Figure 5.10A) and the response elicited to these plasmids. There is almost a threshold type relationship between the amount of antigen available for presentation and the resulting response, especially after boost. Once this threshold is reached, a good immune response is elicited irrespective of the amount of antigen in the system. This reasoning is further supported by the observation that a similar magnitude of NT response was obtained in the first and second *in vivo* experiments despite a 10 fold difference in p 7313 plasmid used for immunisations (i.e. 50 and 5 ng p7313 plasmids used in experiments 1 and 2, respectively). Finally, from the *in vivo* data, there is inconclusive evidence to suggest that the inclusion of the enhancer subunit (present in ii.NT only) resulted in improved promoter potency. However, these results implicate sequences from the initial portion of CMV exon-1 promoter as important in providing enhanced gene expression.

5.4.3. Can the *in vitro* model predict *in vivo* immune responses?

Assessing the immunising dose and promoter dose responses *in vitro* and *in vivo* provided an opportunity to investigate if the *in vitro* CD8+ assay could be predictive of the *in vivo* immune response. When the *in vitro* and *in vivo* immune responses were compared against each other for the immunising dose and promoter dose data, the best correlations were achieved when the post primary *in vivo* time-points were used for correlation (see Tables 5.3 and 5.4). A possible explanation for this is that early on in the *in vivo* immune response (especially day 7 PP), the influenza₃₆₆₋₃₇₄ specific CD8+ T cells may still be clonally expanding in response to the first antigenic stimulus with the memory phenotype not established. Assuming that T cell recruitment is not limiting and that a naïve cytokine milieu exists within the immune micro-environment at this time-point (i.e. no cytokine or T

cell help available from already established T cell populations), the main factor in shaping the immune response is the amount of available antigen present. The response at this stage is not affected by events such as affinity maturation of the T cell repertoire or competition/co-operation effects at the TcR level. Likewise, the *in vitro* bioassay is a one dimensional system which is not complicated by the presence of different types of presenting and responding cell populations. It is responsive to the level of antigen presented which is effectively, in this system at least, determined by the amount of plasmid administered and the amount of antigen produced within the APC. Despite the fact that *in vitro*, the MC57 cells are non professional APC, the *in vitro* bioassay is useful as a guide to determining the early stage primary response *in vivo* but is not suitable for predicting the immune response to a mature memory CD8+ T cell population.

5.5. Conclusions

In this chapter, parallel *in vitro* and *in vivo* experiments were performed in which the influence of immunising DNA dose on resulting immune response was assessed. The main observations were

- That there was a difference in sensitivity between the *in vitro* and *in vivo* experiments, with the *in vivo* dose response studies showing a far greater level of assay sensitivity.
- There was a broad correlation between antigen produced and resulting response *in vitro* but not *in vivo*.
- The immune response observed *in vivo* suggested that there was a lack of correlation between the amount of antigen available for presentation within the APCs and the resulting response after boost.

As a corollary to the dose response studies, the predictive capacity of the *in vitro* assay was assessed. The positive correlations between the *in vitro* data and the early *in vivo* time-points suggested that the *in vitro* assay was useful in determining the early stage primary response *in vivo*.

Chapter 6

Effect of antigen dose on PMDD efficacy following dual plasmid immunisation

6.1. Introduction

In the previous chapter, the pVAC1.NT/pVAC1 dose response was compared *in vitro* and *in vivo*. The correlations obtained suggested that the *in vitro* assay was useful as a guide in determining the early stage primary response *in vivo* but not good for predicting the immune response to a mature memory CD8+ T cell population. A possible explanation for this outcome was that similar to the *in vitro* assay, an early stage *in vivo* response is dominated by its dependence on antigenic load. After this time-point *in vivo*, the immune response moves towards more of a memory phenotype which is influenced more by the behaviour of the memory T cell population than by the amount of antigen available for presentation.

In the previous chapter, an immune response was elicited against the influenza nucleoprotein only. In this chapter, the *in vitro* and *in vivo* models are extended to look at the interaction between two antigens. This was achieved by replacing the non antigen coding plasmid (i.e. pVAC1) with an antigen coding plasmid (i.e. pVAC1.OVAs). In doing so, the effect that a second coding plasmid has on the outcome of the NT CD8+ T cell response is examined. Such an analysis may help to explain better the mechanism of antigenic competition within the APC *in vivo* when more than one plasmid is delivered by PMDD and the effect that this has on overall antigen presentation.

Secondly, the effect that the nature of the antigen produced by pVAC1.OVA has on the NT response was assessed. This was achieved by comparing responses to NT encoding plasmid in the presence of pVAC1.OVAs which secretes the encoded ovalbumin with pVAC1.OVAc which retains the encoded ovalbumin within the cytoplasm or pVAC1. Several studies have demonstrated that altering the cellular location of expressed antigen has quantitative and qualitative effects on immune responses (Boyle *et al.*, 1997; Rush *et al.*, 2002; Torres *et al.*, 1999). Generally, these studies report that following intra-dermal DNA immunisation, membrane bound or secreted forms of antigen favour antibody mediated responses while cytoplasmically localised sources of antigen are more effective at

initiating cellular responses. The influence of cytoplasmically and secreted forms of ovalbumin on the *in vivo* and *in vitro* NT CD8+ response is investigated in this chapter.

6.2. Objective

The relationship between available antigen and resulting CD8+ NT response during dual plasmid immunisation was examined by investigating *in vitro* and *in vivo*

- The effect of a pVAC1.NT/pVAC1.OVAs dose response on resulting CD8+ immune responses.
- The effect that different forms of pVAC1.OVA has on the CD8+ response.

The ability of the *in vitro* CD8+ T cell assay to predict *in vivo* responses was assessed by comparing the *in vitro* and *in vivo* data sets.

6.3. Results

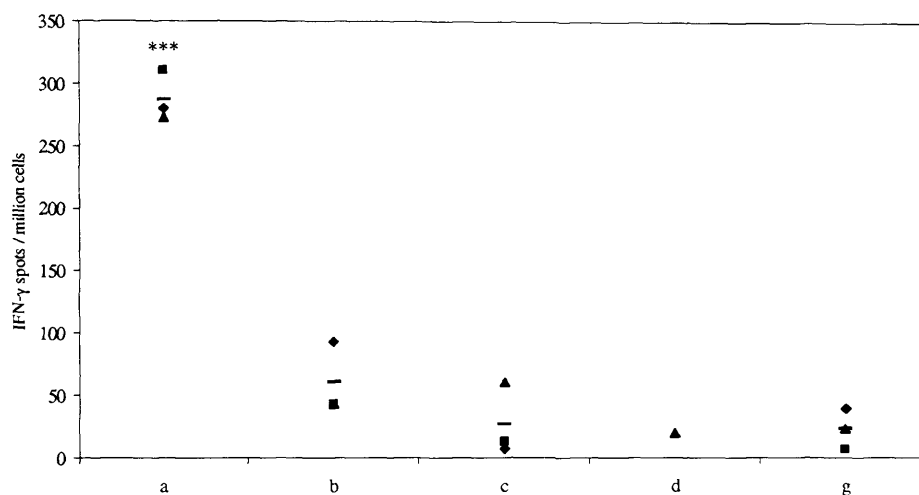
For all experiments, the nomenclature used to denote amount of NT encoding DNA delivered by PMDD is shown in Table 6.1. The *in vitro* results are expressed as number and area of IFN- γ producing cells/million responder cells and the *in vivo* results are expressed as number of IFN- γ producing cells/million cells.

6.3.1. CD8+ restricted T cell responses to pVAC1.NT *in vitro*

For the dose response, cartridges were prepared by mixing pVAC1.NT (in log dilutions from 500–0.5 ng) with variable amounts of pVAC1.OVAs. The total amount of DNA/cartridge was 500 ng. The pVAC1.NT/pVAC1.OVAs dose response was assessed *in vitro* using the CD8+ T cell assay (Figure 6.1). Results show that only the 500 ng group was significantly above control (i.e. pVAC1.OVAs only group). Next, two more batches of

Cartridge composition		
Group name	Theoretical loading pVAC1.NT ng DNA / cartridge	Theoretical loading pVAC1.OVAs ng DNA / cartridge
a	500	0
b	50	450
c	5	495
d	0.5	499.5
e	0.05	499.95
f	0.005	4999.995
g	0	500

Table 6.1. Nomenclature used to denote amount of influenza nucleoprotein and ovalbumin encoding plasmid DNA in cartridge preparations for all *in vitro* and *in vivo* experiments presented in chapter 6.



In vitro pVAC1.NT / pVAC1.OVAs dose response

Figure 6.1. Activation of RAG1.F5 splenocytes by MC57 cells as measured by IFN- γ ELISPOT. MC57s were transfected with pVAC1.NT/pVAC1.OVAs containing cartridges and incubated overnight in a 1:1 ratio with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2. Each symbol (except bars) corresponds to an individual experiment. Bars are the mean of all experiments for each group. For each individual assay, data is mean of triplicate analyses of pooled triplicate transfections. Stars on top of group a represents Dunnet's test comparison of group mean with control group mean (group g) ($p < 0.001$).

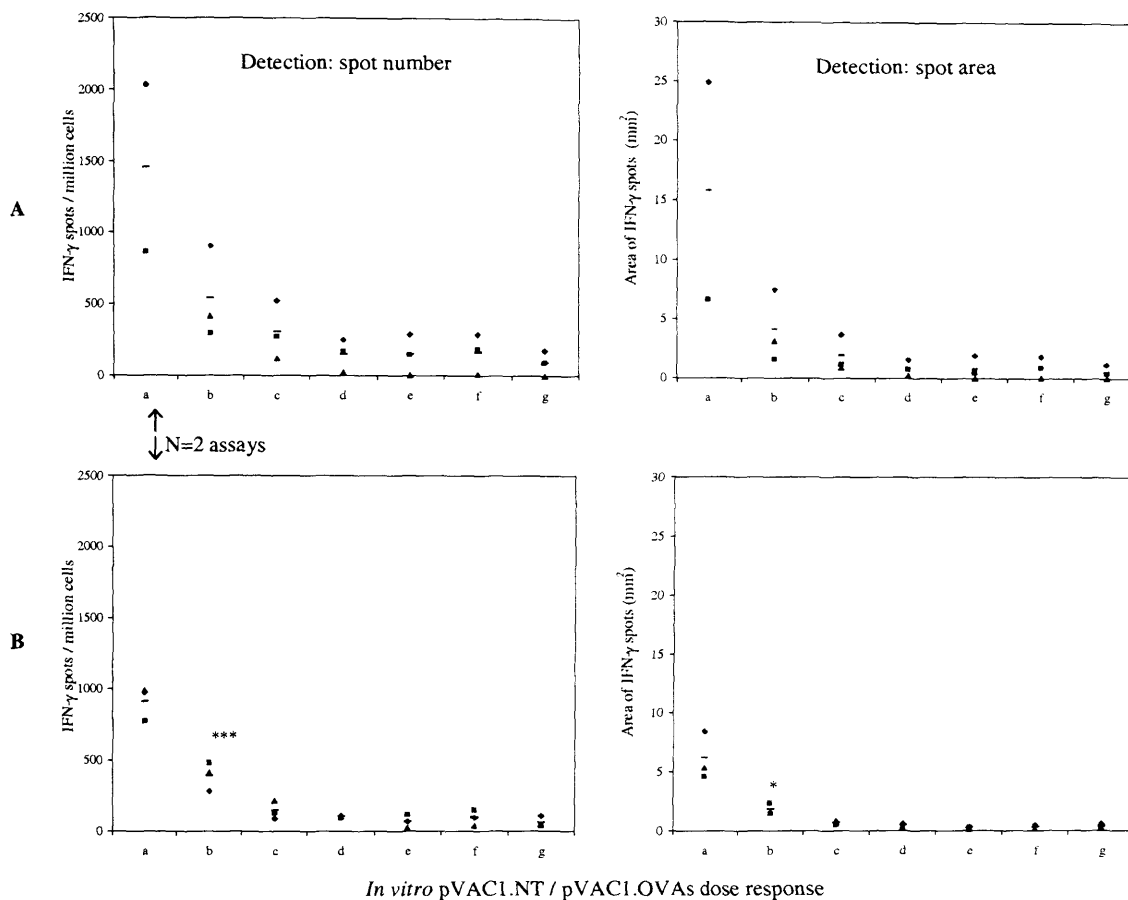


Figure 6.2. Activation of NT specific activated RAG1.F5 splenocytes by MC57 cells measured by IFN- γ ELISPOT. MC57s were transfected with pVAC1.NT/pVAC1.OVAs containing cartridges and incubated overnight in a 1:1 ratio with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2. First and second experiments are shown in rows A and B, respectively. Detection was reported by number (left hand column) and area (right hand column) of IFN- γ producing cells/million cells. Each symbol (except bars) corresponds to an individual experiment. Bars are the mean of all experiments for each group. For each individual assay, data is mean of triplicate analyses of pooled triplicate transfections. Stars on top of groups represent Dunnet's test comparison of group means with corresponding control groups (group g) (* $p < 0.05$, *** $p < 0.001$).

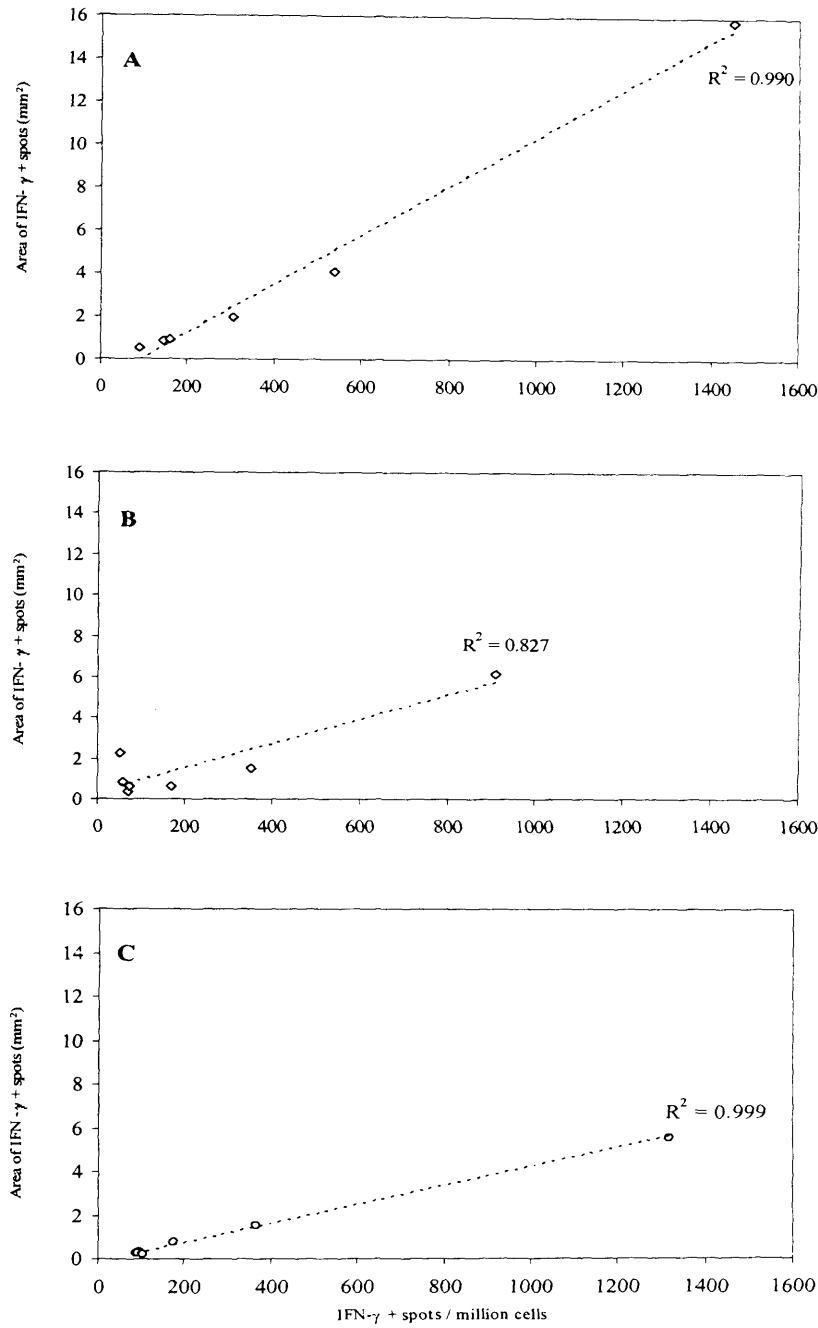


Figure 6.3. Comparison of data reporting methods for the pVAC1.NT/pVAC1.OVAs dose response studies. Panels A and B represent correlation plots of dose response studies shown in Figure 6.2, row A and row B, respectively. Panel C: Correlation plot of all *in vitro* pVAC1.NT/pVAC1.OVAs dose response studies performed (n=8). Mean values of number (x-axis) and area (y-axis) of IFN- γ producing cells/million cells from each group (denoted by horizontal bars in Figures 6.2) was used for correlation and linear trend-line regression coefficient (R^2) was fitted to data.

pVAC1.NT/pVAC1.OVAs cartridges used in parallel *in vivo* experiments (represented in Figure 6.4 and 6.5) were assessed *in vitro*. Detection was reported by number and area of IFN- γ producing cells/million responder cells. Results of the first batch are shown in Figure 6.2, row A. Due to the variability between assays especially at the higher doses of pVAC1.NT, no groups were significantly different from control. Responses above control were evident at the top dose of pVAC1.NT but this group was not tested for significance (n=2 assays at this dose). Similar analyses were performed for the second batch of cartridges. In this dose response, significance was obtained in the 50 ng groups when both number and area of IFN- γ producing cells/million responder cells was used as reporting method (Figure 6.2, row B).

To examine the relationship between number and area of data reporting further, both methods were compared. Regression coefficients of 0.990 and 0.827 were obtained for the first and second batches of cartridges, respectively. When this correlation was performed using the data from all *in vitro* pVAC1.NT/pVAC1.OVAs dose responses (n=8), a R^2 value of 0.999 was obtained (Figure 6.3, panel C).

6.3.2. CD8+ restricted T cell responses to pVAC1.NT *in vivo*

Cartridges containing mixtures of pVAC1.NT/pVAC1.OVAs were assessed in two *in vivo* dose response studies. CD8+ specific T cell responses were measured by IFN- γ ELISPOT and intracellular IFN- γ staining. For the IFN- γ ELISPOT data, results presented are the number of IFN- γ spots in the media + IL-2 + peptide group minus the media + IL-2 group.

For the ELISPOT analysis of the first experiment, significant results were obtained in groups down to 5 ng pVAC1.NT at all time-points (Figure 6.4A). Generally, the magnitude of the IFN- γ response was optimal at day 7 PB before decreasing slightly at day 14 PB. The 500 and 50 ng groups showed the greatest change in IFN- γ production over the time-points studied (e.g. the 50 ng group gave ~140, ~640 and ~400 cytokine producing cells/million cells at day 14 PP and days 7 and 14 PB, respectively). In the repeat experiment (Figure 6.4B), groups immunised with 500 and 50 ng pVAC1.NT were significantly different from

the control group at day 7 and 14 PP. After boost, groups down to 0.5 ng reached significance at day 7 and 14. As in the first experiment, the frequency of cytokine producing cells was generally maximal at day 7 PB. The first two groups in the dose response series (500 and 50 ng groups) showed the greatest difference in frequency of IFN- γ producing cells over the four time-points. The 500 ng pVAC1.NT group yielded ~1330 IFN- γ spots/million cells compared to ~500, ~560 and ~660 at days 7 and 14 PP and day 14 PB, respectively. The lowest amount of pVAC1.NT capable of eliciting an immune response as measured by ELISPOT was 5 ng in the initial experiment compared with 0.5 ng in the repeat. In contrast to the pVAC1.NT/pVAC1 dose responses (Figures 5.4A and 5.5A), there is clear evidence for a linear type relationship between dose of DNA and resulting response.

The results obtained for intracellular IFN- γ staining in the first experiment are represented in Figure 6.5A. Significant results were obtained at all time-points. The magnitude and sensitivity of response was greatest at day 7 PB where groups immunised with 0.5 ng pVAC1.NT were significantly above control. As with the IFN- γ ELISPOT analysis of this experiment, the 500 and 50 ng groups showed the greatest change in frequency of CD8_{high} CD62L_{low} IFN- γ producing cells over the different time-points (e.g. the 500 ng group gave 0.16, 1.33 and 0.5 % CD8_{high} CD62L_{low} IFN- γ producing cells at day 14 PP and days 7 and 14 PB, respectively).

Results of the intracellular IFN- γ staining analysis of the second experiment are shown in Figure 6.5B. In general, the results correlated well with the ELISPOT data except for two notable differences. Firstly, the frequency of CD8_{high} CD62L_{low} IFN- γ producing cells was highest at day 14 PB (500 ng group yielded 0.71 % compared with 0.11, 0.44 and 0.43 % at days 7 and 14 PP and day 7 PB, respectively). Secondly, all groups down to 0.5 ng pVAC1.NT were significantly above control at day 7 PP compared with just the 500 and 50 ng groups in the IFN- γ ELISPOT analysis. Overall, the lowest amount of pVAC1.NT capable of eliciting a response above control as detected by intracellular IFN- γ staining in both experiments was 0.5 ng.

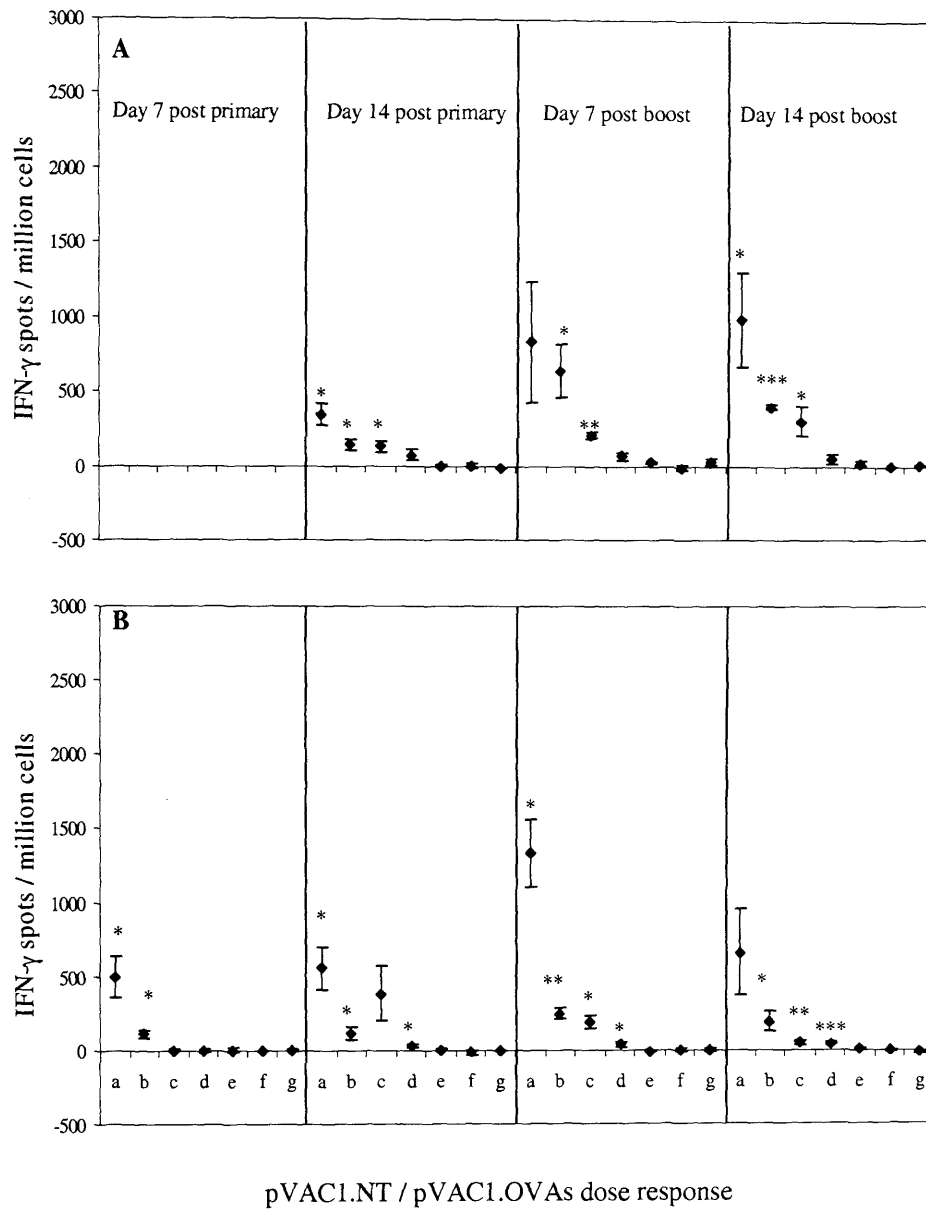


Figure 6.4. NT specific IFN- γ producing CD8+ T cells detected by ELISPOT. Mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1.OVAs dose response and spleens collected at day 14 PP and days 7 and 14 PB in the first experiment (A) and at days 7 and 14 PP and PB in the repeat (B). Prior to ELISPOT analysis, splenocytes were cultured overnight in the presence of media + 50 ng/ml IL-2 or media + 50 ng/ml IL-2 + 100nM NT peptide. Results are mean of individual means \pm SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with pVAC1.OVAs only group (group g) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

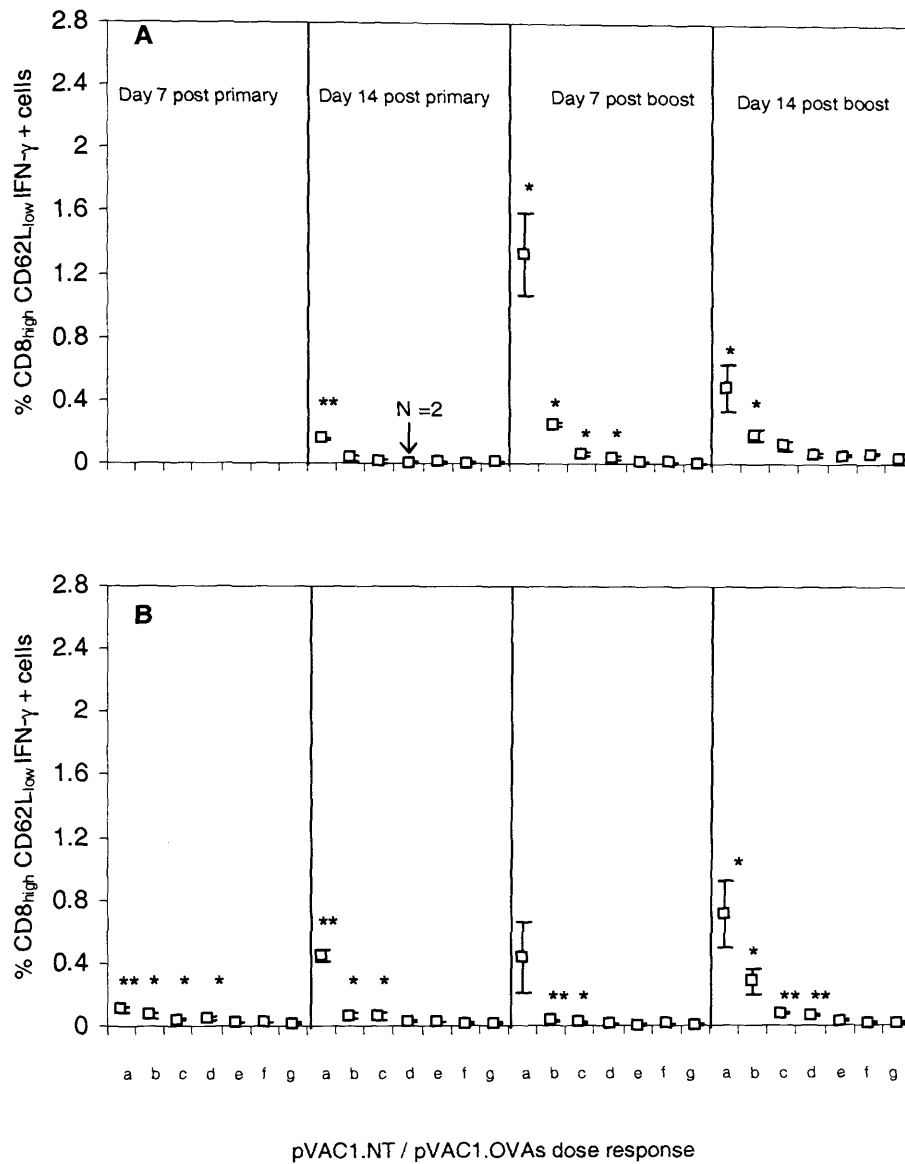


Figure 6.5. Intracellular staining analysis of NT specific CD8+ T cells. Mice were primed and boosted (on day 28) with a pVAC1.NT/pVAC1.OVAs dose response and CD8+ T cell responses detected as NT specific IFN- γ producing cells in splenocytes collected at day 14 PP and days 7 and 14 PB in the first experiment (A) and days 7 and 14 PP and PB in the repeat experiment (B). Splenocytes were incubated with 100 nM NT for 6 hrs and 10 μ g/ml Brefeldin A (last 4 hrs) before staining and flow cytometry analysis. Results are mean of individual means \pm SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with pVAC1.OVAs only group (group g) (* $p < 0.05$, ** $p < 0.01$).

6.3.3. Comparison of T cell responses *in vitro* and *in vivo*

The *in vitro* pVAC1.NT/pVAC1.OVAs dose response data (using number of IFN- γ producing cells/million cells as reporting method) was plotted against the *in vivo* dose response data. For the first batch of cartridges, results showed a good correlation between *in vitro* and *in vivo* data sets (Figure 6.6). The best correlation coefficient was obtained at day 14 PB (R^2 value of 0.965). A similar trend was observed when the *in vitro* IFN- γ ELISPOT and *in vivo* intracellular IFN- γ staining data sets were plotted against each other. Regression coefficients > 0.9 were obtained at each time-point with the best result at day 14 PB (R^2 value of 0.999). The regression coefficients from the *in vitro* IFN- γ ELISPOT/*in vivo* IFN- γ ELISPOT correlation plots for the first and second batches of cartridges are represented in Table 6.2. For both sets of data, good correlations were generally achieved when the *in vitro* data was plotted against data from both the post primary and post boost *in vivo* time-points.

The predictive ability of the *in vitro* bioassay was also assessed by plotting the pVAC1.NT/pVAC1 (see chapter 5) and pVAC1.NT/pVAC1.OVAs dose responses against each other (for the *in vitro* and *in vivo* IFN- γ ELISPOT data sets) and comparing the resulting regression coefficients. For the *in vitro* data, results generated using number of IFN- γ producing cells/million responders as reporting method was used for correlation. When the pVAC1.NT/pVAC1 and pVAC1.NT/pVAC1.OVAs dose responses were compared for all *in vitro* assays performed ($n=11$), an R^2 value of 0.999 was obtained (Figure 6.7). This type of analysis was also performed for the *in vivo* ELISPOT data at each experimental time-point. In contrast to the *in vitro* data, the correlations between the two *in vivo* dose responses for the first batch of cartridges were lower with R^2 values of 0.757, 0.610 and 0.399 obtained at day 14 PP, day 7 PB and day 14 PB, respectively (Figure 6.8). The regression coefficients for the first and second batches of cartridges are compared in Table 6.3. For the second batch of cartridges, correlations between dose responses were also low with the exception of the day 7 PP time-point which yielded a R^2 value of 0.972.

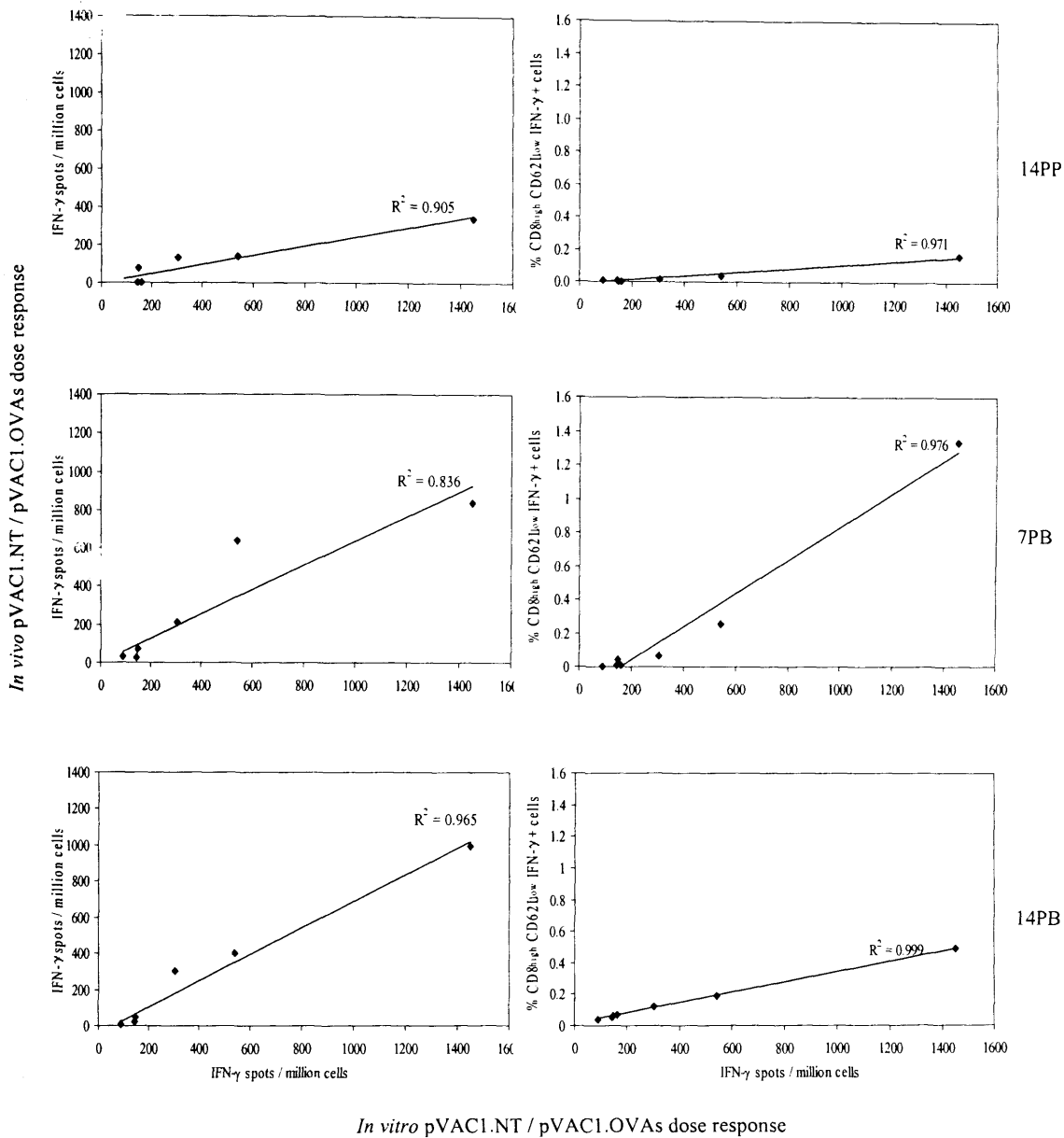


Figure 6.6. Comparison of the *in vitro* and *in vivo* pVAC1.NT/pVAC1.OVAs dose response studies for the first batch of cartridges. The *in vitro* IFN- γ ELISPOT data (Figure 6.2, row A) was plotted against the *in vivo* IFN- γ ELISPOT (Figure 6.4A) and intracellular IFN- γ staining data (Figures 6.5A) and the resulting correlation plots are shown on the left hand and right hand columns, respectively. The dose responses were compared for the day 14 PP, day 7 PB and day 14 PB *in vivo* time-points. Mean values were used for correlation and linear trend-line regression coefficient (R^2) was fitted to data.

R ² values from the <i>in vitro</i> IFN- γ ELISPOT / <i>in vivo</i> IFN- γ ELISPOT correlation plots		
Time-point	Cartridge batch 1	Cartridge batch 2
Day 7 PP	-	0.973
Day 14 PP	0.905	0.636
Day 7 PB	0.836	0.953
Day 14 PB	0.965	0.989

Table 6.2. Comparison of the *in vitro* and *in vivo* pVAC1.NT/pVAC1.OVAs dose response studies for the first and second batch of cartridges. R² values were obtained by plotting *in vitro* IFN- γ ELISPOT data against the *in vivo* IFN- γ ELISPOT data.

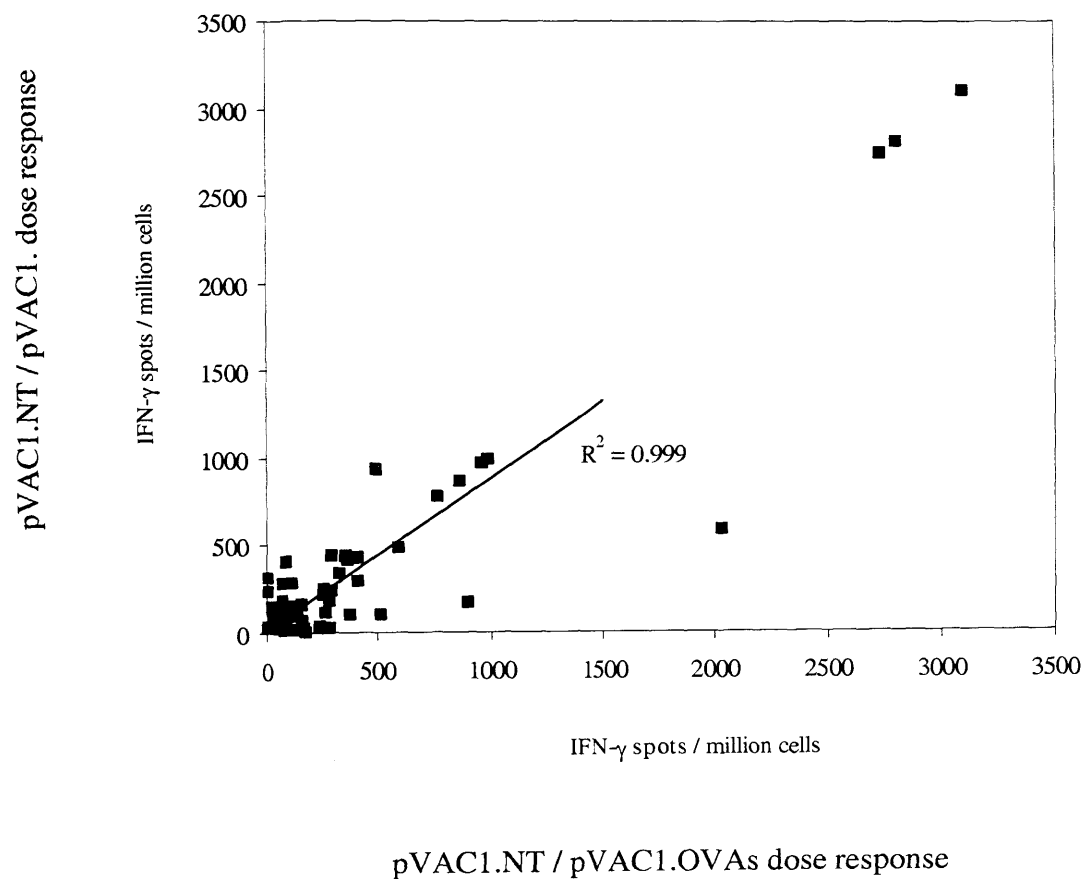


Figure 6.7. Comparison of all *in vitro* pVAC1.NT/pVAC1.OVAs and pVAC1.NT/pVAC1 dose responses performed (n=11). For each dose response, mean values were used for correlation and linear trend-line regression coefficient (R^2) was fitted to data.

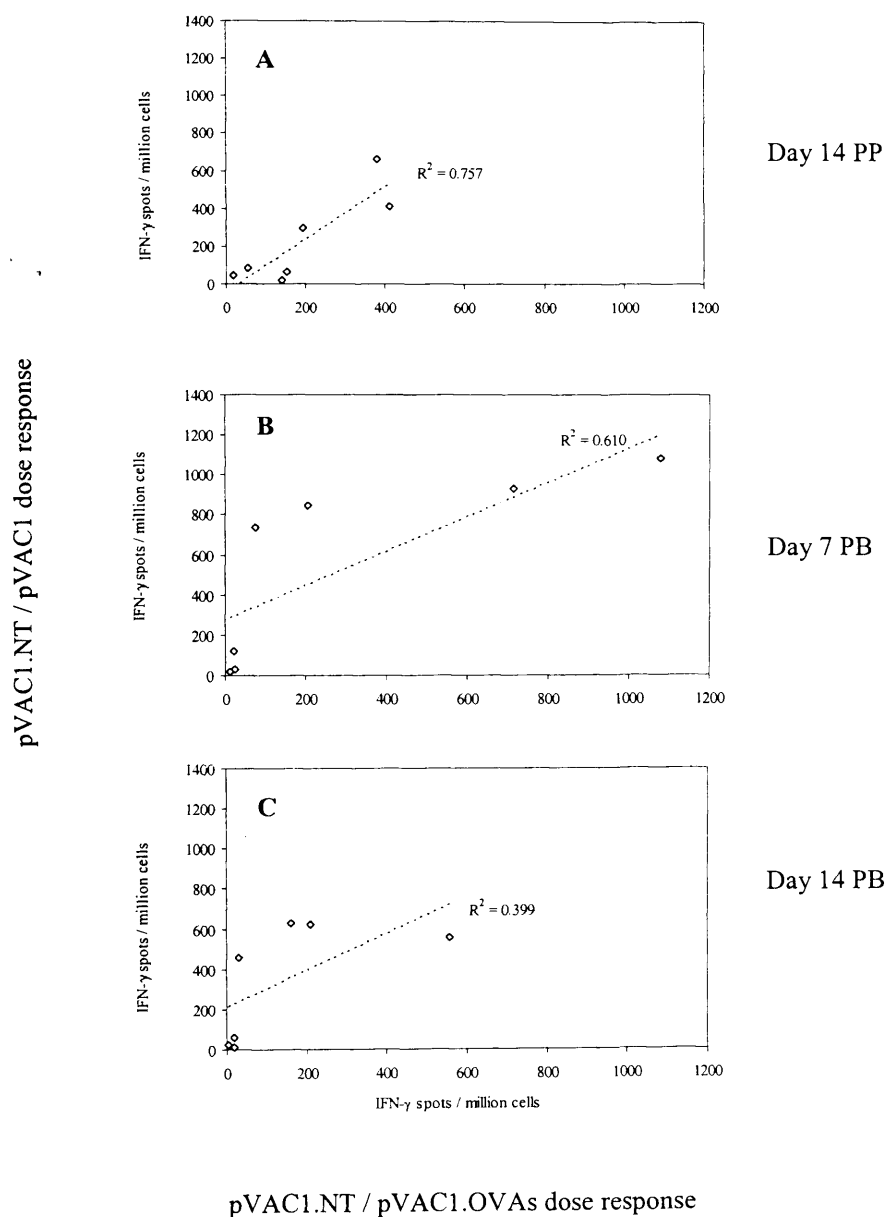


Figure 6.8. Comparison of *in vivo* pVAC1.NT/pVAC1.OVAs and pVAC1.NT/pVAC1 dose responses for the first batch of cartridges. Data from IFN- γ ELISPOT (shown in Figures 6.1A and 5.4A) was used for correlation. Panels A-C: Correlation plots of day 14 PP, day 7 PB and day 14 PB data. Mean values were used for correlation and linear trend-line regression coefficient (R^2) was fitted to data.

R ² values from the <i>in vivo</i> pVAC1.NT/ pVAC1 and pVAC1.NT/ pVAC1.OVAs IFN-γ ELISPOT correlation plots		
Time-point	Cartridge batch 1	Cartridge batch 2
Day 7 PP	-	0.972
Day 14 PP	0.757	0.525
Day 7 PB	0.610	0.558
Day 14 PB	0.399	0.775

Table 6.3. Comparison of *in vivo* pVAC1.NT/pVAC1.OVAs and pVAC1.NT/pVAC1 dose responses for the first and second batches of cartridges. Data from IFN-γ ELISPOT was used for correlation. Mean values were used for correlation and linear trend-line regression coefficient (R²) was fitted to data.

6.3.4. Effect of expressed Ovalbumin on the NT response

MC57 cells were transfected by PMDD with cartridges containing 50 ng pVAC1.NT and 450 ng of pVAC1, pVAC1.OVAc or pVAC1.OVAs and the resulting NT response analysed using the *in vitro* assay. MC57 cell cultures were treated with 20 ng/ml of IFN- γ for 24 hrs prior to the assay in an effort to increase assay sensitivity by up-regulation of surface expressed MHC I. These cells were then compared with normal MC57 cells for their ability to stimulate NT specific IFN- γ secretion (Figure 6.9A). Cells with up-regulated MHC I expression proved superior in their antigen presenting capacity compared to normal MC57s with each PMDD group showing a significant increase in IFN- γ secretion (between ~180-270 %). In contrast, both positive control groups (i.e. peptide pulsed cells) yielded similar results. This assay was repeated on two further occasions (IFN- γ treated) and the results from all three assays are shown in Figure 6.9B. Although, the magnitude of the IFN- γ response to the different plasmid combinations was increased over that usually observed with normal MC57 cells, there was no significant difference between any of the PMDD transfected groups. The effect of IFN- γ treatment on expression of MHC I Kd surface expression is represented in Figure 6.9C.

To directly compare the *in vitro* model with immune responses *in vivo*, cartridges containing the different plasmid combinations were assessed both *in vitro* and *in vivo*. In these experiments, the MC57 cells were not pre-treated with IFN- γ prior to use in the *in vitro* assay. Results revealed no difference in IFN- γ secretion between any of the PMDD transfected groups (Figure 6.10). The *in vitro* results contrasted markedly with those obtained *in vivo* (Figure 6.11A). At each time-point, the pVAC1.NT/pVAC1 group elicited the strongest response yielding ~165 and ~1080 spots/million cells at day 7 PP and 7 PB, respectively. After boost, all groups were significantly different from each other. When this analysis was repeated *in vivo* using a separate batch of cartridges, a similar trend emerged (Figure 6.11B). Again, the greatest response was obtained in the pVAC1.NT/pVAC1 groups at both time-points (~250 and 1125 spots/million cells at day 7 PP and 7 PB, respectively) with this group attaining significance over the pVAC1.NT/pVAC1.OVAc and pVAC1.NT/pVAC1.OVAs groups at day 7 PB.

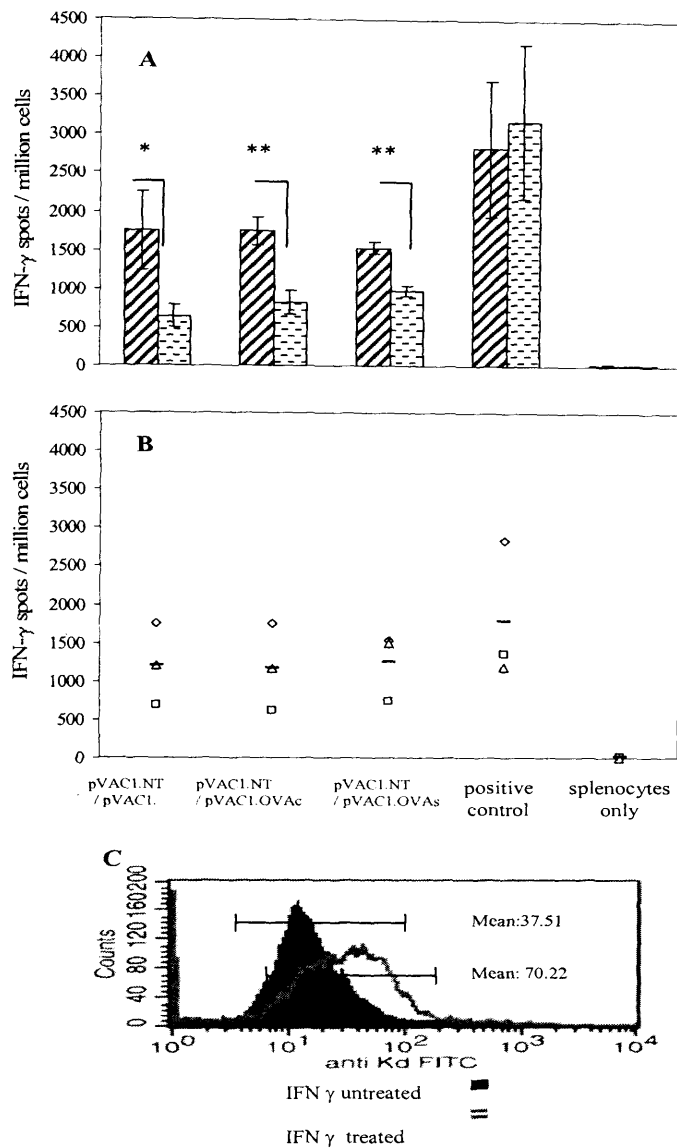


Figure 6.9. Effect of dual plasmid immunisation measured *in vitro* by IFN- γ ELISPOT. MC57 cells were transfected with different plasmid combinations and incubated overnight in a 1:1 ratio with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2. Panel A: Comparison of IFN- γ treated (diagonal bars) and untreated (horizontal bars) MC57s in stimulating a NT specific response. Data is from one experiment and is mean (\pm standard deviation) of triplicate analyses of pooled triplicate transfections. Stars represent Student's T-test comparison between corresponding groups (* $p < 0.05$, ** $p < 0.01$). Panel B: Effect of IFN- γ treated MC57 cells in stimulating NT specific IFN- γ secretion in the *in vitro* CD8 $^+$ T cell assay. Each symbol (except bars) represents an individual experiment. Bars are the mean of all experiments for each group. For each individual experiment, data is mean of triplicate analyses of pooled triplicate transfections. Panel C: Representative data showing the effect of IFN- γ treatment (20 ng/ml for 24 hrs) on MHC I Kd expression.

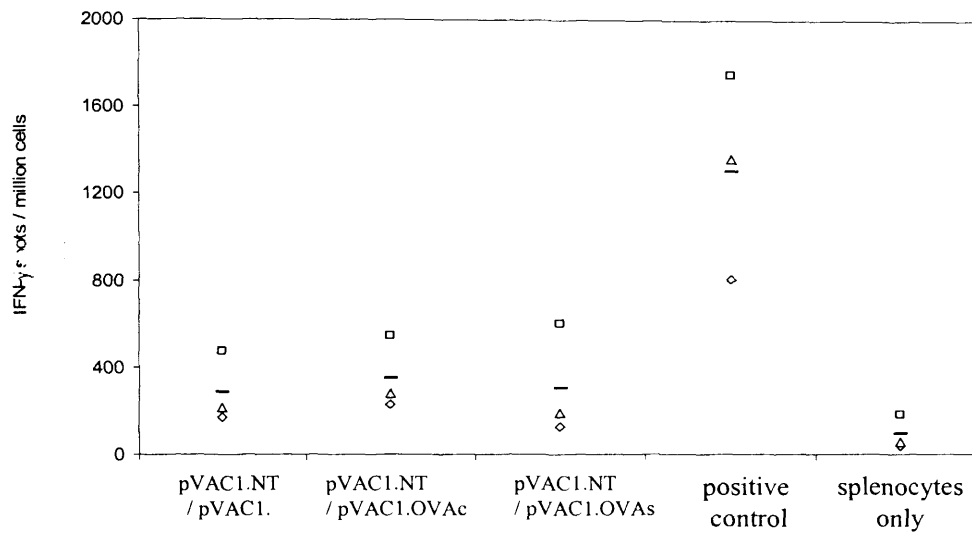


Figure 6.10. Assessing the effect of dual plasmid immunisation on the NT response *in vitro* using the CD8⁺ T cell assay. MC57 cells were transfected with different plasmid combinations and incubated overnight in a 1:1 ratio with 1×10^5 activated RAG1.F5 splenocytes and 200 ng/ml IL-2 prior to IFN- γ ELISPOT analysis. Each symbol (except bars) represents individual experiments. Bars are the mean of all experiments for each group. For each individual experiment, data is mean of triplicate analyses of pooled triplicate transfections.

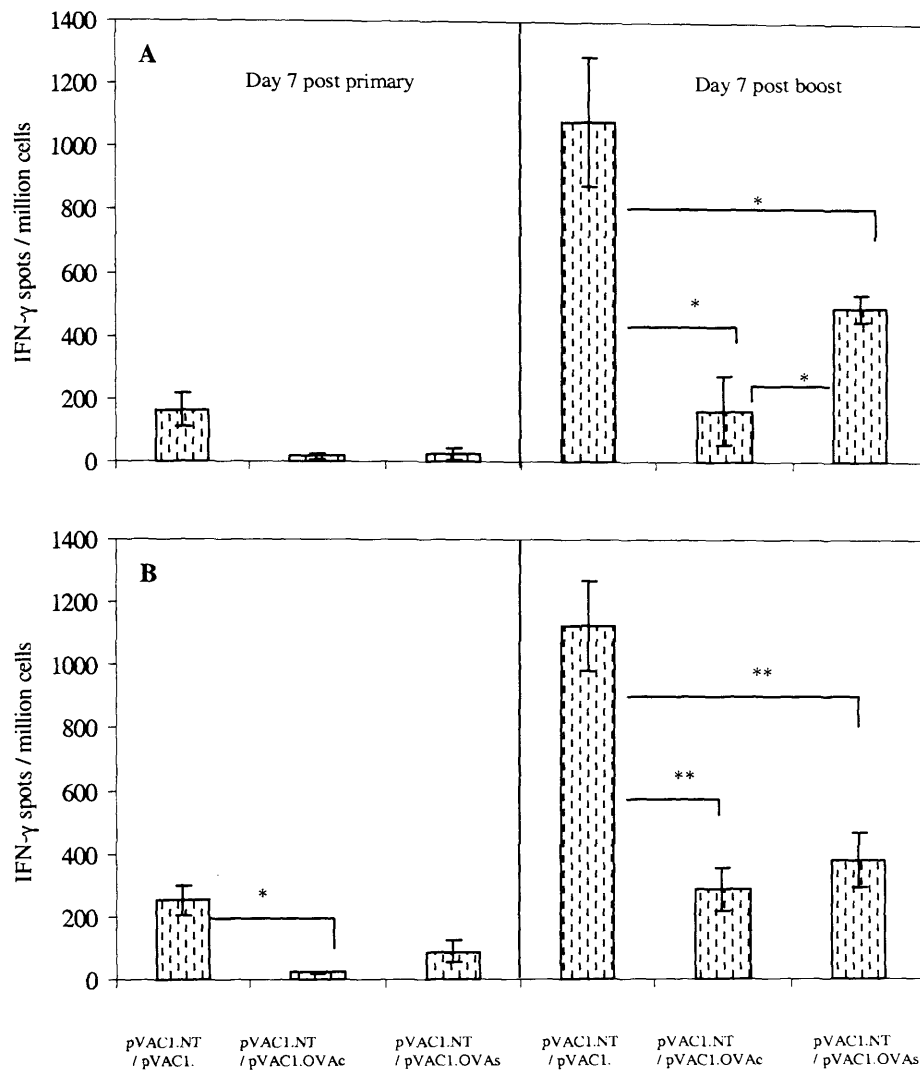


Figure 6.11. Assessing the effect of dual plasmid immunisation on the NT response *in vivo* by IFN- γ ELISPOT. Mice were primed and boosted (on day 28) with different plasmid combinations and spleen cells were collected at day 7 PP and PB. Purified splenocytes were cultured overnight in media + 50 ng/ml IL-2 or media + 50 ng/ml IL-2 + 100nM NT peptide prior to ELISPOT analysis. Results are mean of individual means \pm SEM of 3 mice/group. Initial and repeat experiments are shown in panels A and B, respectively. Stars represent Student's T-test comparison between corresponding groups (* $p < 0.05$, ** $p < 0.01$).

6.4. Discussion

6.4.1. Effect of co-immunisation on the NT response *in vitro*

The effect of co-immunisation on the NT response was investigated *in vitro* in the form of a pVAC1.NT/pVAC1.OVAs dose response. Results showed that the presence of pVAC1.OVAs plasmid appeared to have no effect on the magnitude or the sensitivity of the NT response in the various dose response groups. Furthermore, the sensitivity of both dose responses was similar at 50 ng DNA (second batch of pVAC1.NT/pVAC1.OVAs cartridges only). The same result was obtained when MC57 cells were transfected with pVAC1.NT in the presence of pVAC1.OVAs, pVAC1.OVAc or pVAC1. These results suggest that there is no significant competition at the level of transcription, processing and MHC loading at least in the MC57 fibrosarcoma cell line.

The effect of MC57 IFN- γ stimulation on resulting NT response *in vitro* was also investigated. IFN- γ is known to enhance MHC expression by stimulating the rate of transcription of class I and II genes in a wide range of cells (Rosa *et al.*, 1988; Pace *et al.*, 1983). In this study, there was a significant increase in the magnitude of the NT response in groups containing MC57 cells treated with IFN- γ . There was, however, no difference between any of the plasmid combinations. Interestingly, although the PMDD treated groups reported an overall increase in the NT response in cells pre-treated with IFN- γ , the positive control groups in these experiments (i.e. peptide pulsed cells) did not. This suggests that the impact of IFN- γ pre-treatment in this cell line was more to do with the enhancement of proteins involved in antigen processing and presentation, namely β_2 microglobulin and the TAP heterodimer (Mutlu *et al.*, 1991; Schiffer *et al.*, 2002) than the up-regulation of surface MHC 1.

6.4.2. The NT and OVA relationship *in vivo*

The influence that pVAC1.OVAs has on the NT CD8+ response is clearly demonstrated in the *in vivo* dose response and co-immunisation studies. The magnitude of the

pVAC1.NT/pVAC1.OVAs and pVAC1.NT/pVAC1 dose responses is markedly different (see Figure 5.4 and 5.5). The threshold type association between plasmid administered and resulting response observed in the pVAC1.NT/pVAC1 dose response after boost is replaced by a more linear type of relationship here (at all time-points). A possible explanation for this difference may be that in a pVAC1.NT/pVAC1 dose response, an immune response is elicited against the influenza nucleoprotein only. There are no limiting factors associated with the APC or the T cell repertoire such as access to cytokines, access to the DC surface, number of antigen loaded DC and T cell affinity maturation which may impede the resulting response. After boost, memory T cells react vigorously to NT antigen presented, regardless of the amount. Consequently, there is a threshold type relationship between antigen dose and resulting response.

For the pVAC1.NT/pVAC1.OVAs dose response, the difference in magnitude of the influenza₃₆₆₋₃₇₄ specific CD8+ T cell subset between the top dose and lower doses is clear. The increase in magnitude of the NT secondary response only occurs in the top dose (i.e. group containing pVAC1.NT only) with all other groups remaining either equal to, or only marginally higher, than the equivalent primary response groups. The data indicates that a second coding plasmid, pVAC1.OVAs, interferes effectively against NT in launching an immune response. This interference, or competition, may occur at one or more levels.

6.4.2.1. Intracellular competition between OVA and NT

The down regulation of the NT response may be due to intracellular competition between the NT and OVA plasmids within the APC *in vivo*. This view is supported by the data from the co-immunisation studies (Figure 6.11). The primary finding was that mice immunised with pVAC1.NT/pVAC1 showed the greatest NT specific CD8+ response after primary and boost immunisation. A lesser response was elicited when NT encoding plasmid was administered in the presence of the ovalbumin encoding plasmids. Intracellular competition is also consistent with investigations by Manoj *et al.* (2003) who reported significant reductions in specific antigen response with the use of a dual plasmid immunisation strategy or a bicistronic vector compared to single plasmid immunisation. Delivering

pVAC1.NT in the presence of pVAC1.OVAs appeared to have a less suppressive effect on the NT CD8+ response compared with co-delivering pVAC1.NT and pVAC1.OVAc. As pVAC1.NT and pVAC1.OVAc encode predominately cell retained antigen, this result may reflect the competition effect between NT and OVAc for entry into the MHC I department. Indeed, if the major cell type transfected were skin DC (i.e. the Langherhans cells), competition would not only occur at the MHC loading step but also at the protein synthesis stage prior to this. In contrast, the pVAC1.OVAs plasmid encodes for a predominately secreted form of OVA leading to reduced level of antigenic competition compared with OVAc. Furthermore, this may result in a superior helper effect mediated by secreted ovalbumin derived CD4+ T cells. A non specific mechanism of action may include the secretion of cytokines such as IL-2 and IFN- γ which may promote the responsiveness of the CD8+ NT cells. Additionally, OVA CD4+ T cells may provide help by the activation of the APC, a mechanism referred to by Wodarz and Jansen (2001) as CD4-APC-CD8 help. This pathway leads to IL-12 secretion by activated DC which helps to promote a Th1 phenotype and is important for priming and maintenance of the CD8+ response (Cella *et al.*, 1996).

However, this explanation is not entirely consistent with previous data on the nature of the OVAs response. In single plasmid immunisations, several studies have demonstrated that the cellular location of expressed antigen quantitatively and qualitatively affects the immune responses (Boyle *et al.*, 1997; van Drunen Littel-van den Hurk *et al.*, 1998; Lewis *et al.*, 1999; Higgins *et al.*, 2000). Indeed for intra-dermal gene gun immunisation, it is widely acknowledged that secreted forms of antigen result in the formation of a humoral response with a prominent Th2 type antibody profile (Scheiblhofer *et al.*, 2001; Kaur *et al.*, 2002). Therefore, the expectation would be that NT/OVAs co-immunisation would create an immune micro-environment with a Th2 type profile (relative to NT/OVAc co-immunisation) resulting in a reduced CD8+ NT response. The fact that this did not happen together with the *in vitro* data suggests that an alternative mechanism may be wholly or partially responsible for the suppressive effects of OVA on the CD8+ NT response.

6.4.2.2. Clonal competition

Antigenic competition between the NT and the OVA response may also occur at the T cell level. The competition may be specific (e.g. T cell-T cell inhibition) or non-specific (e.g. access to cytokines) in nature. Following immunisation with pVAC1.NT and pVAC1.OVAs, the NT and OVA responses are predominately focused on their respective immunodominant epitopes which are of a different specificity (in terms of antigenic determinant and MHC restriction). There is a lot of evidence suggesting that T cells of different specificity do not compete with each other for DC access and T cell activation (Probst *et al.*, 2002; Smith *et al.*, 2000; Creusot *et al.*, 2003a; Creusot *et al.*, 2003b). Therefore, it is unlikely that specific T cell competition occurs.

Alternatively, it is possible that competition for non specific factors around the DC-T cell cluster takes place. These include access to cytokines such as IL-2 (Wodarz and Jansen, 2001), IL-7 and IL-15 (Ge *et al.*, 2004) and IL-12 (Eberl *et al.*, 1996) which are necessary for activation and proliferation. This may provide an explanation for the lower NT responses obtained in the pVAC1.NT/pVAC1.OVAs dose responses, especially considering the possibility that the NT and OVAs antigens are presented on different DCs. There is also the likelihood that, following pVAC1.NT and pVAC1.OVA co-immunisation, the number of DC available for stimulation in the secondary response is insufficient to sustain activation of both memory subsets (Lanzavecchia *et al.*, 2002). In support of this hypothesis as a possible mechanism to explain the suppressive effect of OVA on NT, it is noteworthy that the lowest amount of pVAC1.NT capable of eliciting a significant response in both types of dose responses was similar (0.5 ng). It was the magnitude and not the sensitivity of the NT response which was reduced in the pVAC1.NT/pVAC1.OVAs dose responses suggesting a level of competition for factors important for either T cell activation and/or proliferation. The functional relationship between these T cell repertoires and the implications for the resulting immune response is investigated further in chapter 7.

6.4.3. Can the *in vitro* model predict *in vivo* immune responses?

For the pVAC1.NT/pVAC1 dose response, the best correlations were achieved when the *in vitro* model was compared with the early *in vivo* time-points (see section 5.4.3). Here, the ability of the *in vitro* CD8⁺ T cell assay to predict *in vivo* responses was assessed under conditions of dual plasmid immunisation. For the pVAC1.NT/pVAC1.OVAs dose response, positive correlations were generally achieved when the *in vitro* data was compared against the *in vivo* data at all time-points, post primary and post boost. This difference in trend is a consequence of the differing nature of the two dose responses *in vivo* (see section 6.4.2). The pVAC1.NT/pVAC1 dose response exhibits a threshold type relationship between dose and resulting response after boost and this is reflected in the lesser correlations achieved between the *in vitro* assay and the post boost *in vivo* time-points. In contrast, there is a linear relationship between dose and response for the *in vivo* pVAC1.NT/pVAC1.OVAs dose response at all time-points. This may be due to the competitive interaction between the NT and OVA responses, especially after boost. The result of this was that good correlations were obtained when the *in vitro* data was compared against the *in vivo* data at all time-points.

This result superficially suggests that the *in vitro* model is useful in predicting the *in vivo* response to NT under conditions of dual plasmid immunisation. However, interpreting the data in this way is misleading. Another approach is to plot the pVAC1.NT/pVAC1 and pVAC1.NT/pVAC1.OVAs dose responses against each other (*in vitro* and *in vivo*) and compare the regression coefficients of these plots (Figures 6.7, 6.8 and Table 6.3). The *in vitro* result from this type of analysis ($R^2 = 0.999$) suggested that the response elicited by RAG1.F5 splenocytes to pVAC1.NT was similar regardless of whether MC57s were transfected with cartridges containing the pVAC1.NT/pVAC1 or pVAC1.NT/pVAC1.OVAs plasmids. This was supported by the comparability of the sensitivity data obtained for both sets of dose responses (i.e. 50 ng pVAC1.NT) and was further confirmed by the finding that the *in vitro* assay revealed no difference in NT specific responses when MC57 cells were transfected with pVAC1.NT in the presence of pVAC1.OVAs, pVAC1.OVAc or pVAC1. When the dose responses were compared *in*

vivo, a different trend emerged. With the exception of the day 7PP *in vivo* time-point ($R^2=0.972$ for the second experiment), the correlations were poor. Separately, when the different plasmid combinations were assessed, the response to NT in the pVAC1.NT/pVAC1 group was much greater than the response to NT in the pVAC1.NT/pVAC1.OVAs and pVAC1.NT/pVAC1.OVAc groups. Together, these results clearly indicate that the *in vivo* response is influenced by the co-immunising plasmid.

When the *in vitro* and *in vivo* data were compared in these two ways, it is evident that the *in vitro* model is useful in predicting the early stage primary response to pVAC1.NT *in vivo* (for both dose responses) for reasons explained in section 5.4.3. However, *in vivo* immune responses to NT were strongly affected by the presence or absence of a second antigen, while in contrast *in vitro* responses did not share this effect.

6.5. Conclusions

During this and the preceding chapter, the relationship between available antigen and resulting CD8+ NT response during single and dual plasmid immunisation was examined *in vitro* and *in vivo*. The primary findings were that

- The *in vitro* assay revealed no difference in NT specific responses when MC57 cells were transfected with cartridges containing the pVAC1.NT/pVAC1 or pVAC1.NT/pVAC1.OVAs dose response or when MC57 cells were transfected with pVAC1.NT in the presence of pVAC1.OVAs, pVAC1.OVAc or pVAC1.
- The *in vivo* immune response to NT was strongly affected by the presence or absence of a second coding antigen with the presence of OVAs resulting in the inhibition of the NT response. Furthermore, the data suggests that the cellular location of the antigen produced by pVAC1.OVA may affect the NT response *in vivo*.

The effect of NT and OVA co-immunisation in shaping the CD4+ and CD8+ T cell response and the co-operation/competition between these immune subsets is discussed in the following chapter.

Additionally, the predictive capacity of the *in vitro* model was assessed by comparing the *in vitro* and *in vivo* data. Results suggest that the *in vitro* bioassay is useful as a guide to determining the early stage primary response *in vivo* (for single and dual immunisations) but does not accurately represent the immune response to a mature memory CD8+ T cell population.

Chapter 7

CD8+ T cell competition and co-operation

7.1. Introduction

The analyses described in the previous chapter highlighted the contribution of pVAC1.OVA in shaping the immune response to pVAC1.NT. This was particularly evident after boost, where the presence of pVAC1.OVA resulted in a much reduced NT response compared with immunisation with pVAC1.NT only. The cause of the reduction in NT response and the mechanisms which may have led to this are investigated further in this chapter.

Under conditions of dual plasmid immunisation, the immune responses to the different antigens may interact negatively with each other (i.e. antigenic competition) leading to a focusing of the immune response towards a particular epitope on a particular antigen. Alternatively, both immune responses may interact positively (i.e. antigen co-operation) resulting in a broad and comprehensive response to both antigens. It is most likely however, that following dual plasmid immunisation, both phenomena occur in unison, with the resulting immune response a reflection of the balance achieved between both immune mechanisms.

Antigenic competition, in which responses to one antigenic determinant dominates the immune responses to other determinants is central to the concept of immunodominance (Rammensee *et al.*, 1993). Several factors are known to contribute to immunodominance such as the processing and presenting capacity of the APC (Sercarz *et al.*, 1993), affinity of synthesised peptide for APC MHC I (Campos-Lima *et al.*, 1997) and competition between T cell populations for response to antigen (Smith *et al.*, 2000; Butz and Bevan, 1998a; Sandberg *et al.*, 1998). Intraclonal and interclonal T cell competition is described in more detail in Section 1.3.5.2. Although the preponderance of reports are associated with CD8+ T cell competition (Lawson *et al.*, 2001; Grossmann *et al.*, 2001; Palmowski *et al.*, 2002), there is also evidence of competition between CD4+ T cells (Savage *et al.*, 1999; Rees *et al.*, 1999).

In addition to the competition effects observed in the previous *in vivo* analyses, antigenic co-operation is likely to play a role in shaping the NT response (Creusot *et al.*, 2003a). Antigenic co-operation in the form of CD4–CD8+ T cell co-operation studies have yielded important information. There is clear evidence, for example, that class I and class II epitopes need to be presented on the same APC for the generation of CTL immunity (Bennett *et al.*, 1997). Activation of APC expressed CD40 by CD4+ T cell expressed CD40L appears to be the main trigger in licensing the APC to stimulate CD8+ T cell proliferation (Schoenberger *et al.*, 1998; Ridge *et al.*, 1998) although this mechanism of CD8+ T cell activation has been questioned (Bourgeois *et al.*, 2002). CD4+ T cells also assist CD8+ immunity by secretion of cytokines such as IL-2, which promote the responsiveness of the CD8+ T cells and augment CTL activation (Wodarz and Jansen, 2001). Numerous studies have documented the importance of CD4+ T cells in the maintenance of the CD8+ T cell response during chronic viral infection (Matloubian *et al.*, 1994; Cardin *et al.*, 1996; Belz *et al.*, 2002; Kostense *et al.*, 2002). In addition to the CD4–CD8+ T cell co-operation models mentioned here, there is also evidence suggesting the existence of CD4–CD4+ (Gerloni *et al.*, 2000; Creusot *et al.*, 2003a) and CD8–CD8+ (Sherritt *et al.*, 2000) T cell co-operation *in vivo*.

In this chapter, the effects of antigenic competition and co-operation on the NT and OVA immune responses following PMDD are investigated. The analysis is predominately focused on the CD8+ NT response but the effect of NT and OVA co-immunisation on the resulting OVA response is considered also. Each of the analyses incorporate another aspect of PMDD co-immunisation which arose from the *in vitro in vivo* correlation studies, namely, the effect of OVA intracellular localisation on resulting immune response (see section 6.4.2.1).

7.2. Objective

To investigate the role of antigenic competition and co-operation in shaping the NT and OVA immune responses following co-immunisation of NT plasmid with cytoplasmically localised or secreted OVA encoding plasmid. Investigations include

- The effect of T cell memory on the resulting NT and OVA responses.

by pre-immunisation with pVAC1.NT or pVAC1.OVA only.

by dose response of the co-immunised plasmid (i.e. pVAC1.OVA).

- The effect of antigen linkage on the resulting NT and OVA responses.

7.3. Results

The nomenclature used to denote plasmid composition of the DNA cartridges used in this chapter is described in Table 7.1.

7.3.1. Effect of T cell memory

The effect of T cell memory on NT and OVA responses was investigated in two ways. Firstly, a pre-existing NT or OVA immune response was established in C57BL/6 mice by immunising with pVAC1.NT or pVAC1.OVA only. These mice were then immunised with groups containing NT/empty, NT/OVAc or NT/OVAs plasmid (referred to as groups 1, 2 and 3, respectively). Secondly, mice were primed and boosted with cartridges containing different amounts of OVAc or OVAs plasmid combined with a constant amount of NT plasmid. For the first set of experiments, CD8+ NT and CD8+ and CD4+ OVA responses were assessed while for the repeat experiments, both CD8+ and CD4+ NT and OVA responses were monitored.

Cartridge composition	
Group name	Theoretical loading (ng DNA / cartridge)
1	50 ng pVAC1.NT + 450 ng pVAC1
2	50 ng pVAC1.NT + 450 ng pVAC1.OVAc
2 UL	50 ng pVAC1.NT + 450 ng pVAC1.OVAc (unlinked DNA cartridge)
2.1	50 ng pVAC1.NT + 50 ng pVAC1.OVAc + 400 ng pVAC1
2.2	50 ng pVAC1.NT + 5 ng pVAC1.OVAc + 445 ng pVAC1
3	50 ng pVAC1.NT + 450 ng pVAC1.OVAs
3 UL	50 ng pVAC1.NT + 450 ng pVAC1.OVAs (unlinked DNA cartridge)
3.1	50 ng pVAC1.NT + 50 ng pVAC1.OVAs + 400 ng pVAC1
3.2	50 ng pVAC1.NT + 5 ng pVAC1.OVAs + 445 ng pVAC1
4	50 ng pVAC1.NT + 450 ng pVAC1.RNG
5	50 ng pVAC1.NT + 450 ng pVAC1.HPV

Table 7.1. Nomenclature used to denote amount of influenza nucleoprotein encoding plasmid, ovalbumin encoding plasmid and control plasmid DNA in cartridge preparations for all *in vitro* and *in vivo* experiments presented in chapter 7.

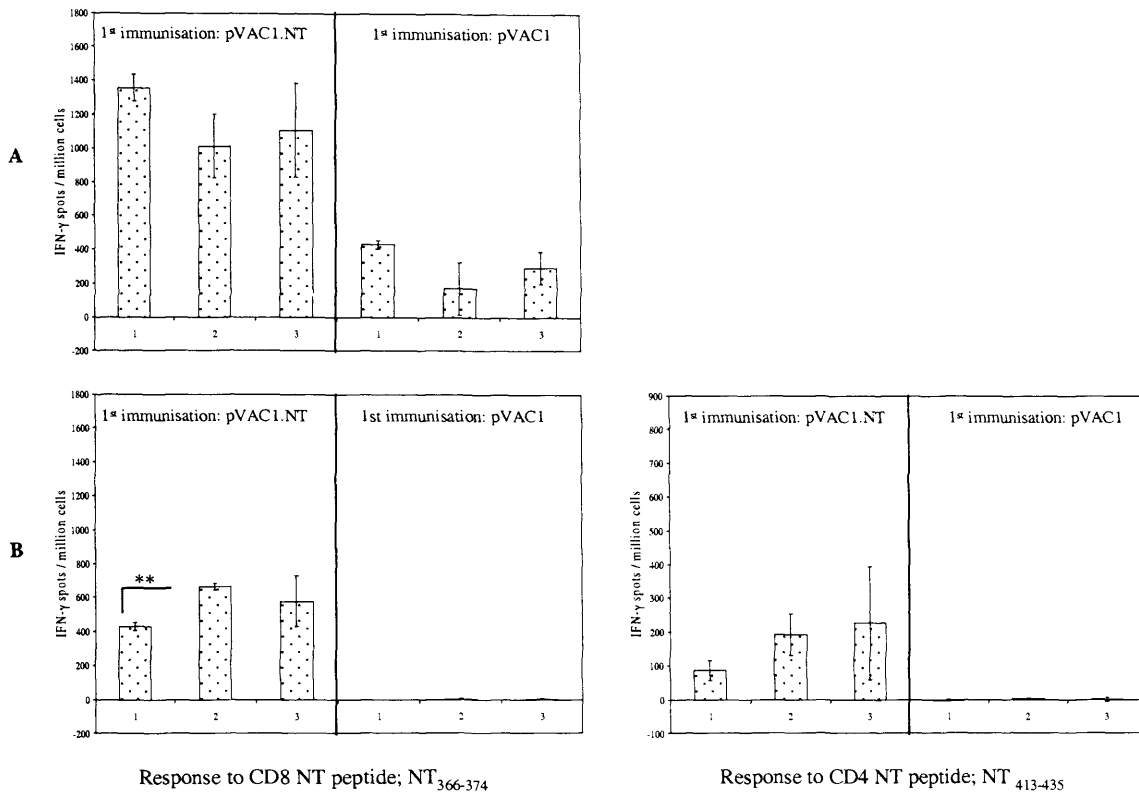


Figure 7.1. NT specific IFN- γ producing CD8+ and CD4+ T cells detected by ELISPOT. Mice were primed with pVAC1.NT or pVAC1 and boosted on day 28 with cartridges containing NT/empty (group 1), NT/OVAc (group 2) or NT/OVAs (group 3). Spleens were collected for analysis at day 7 PB. Initial and repeat experiments are shown in panels A and B, respectively. For ELISPOT analysis, results are reported as mean of individual means \pm SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with corresponding group (** $p < 0.01$).

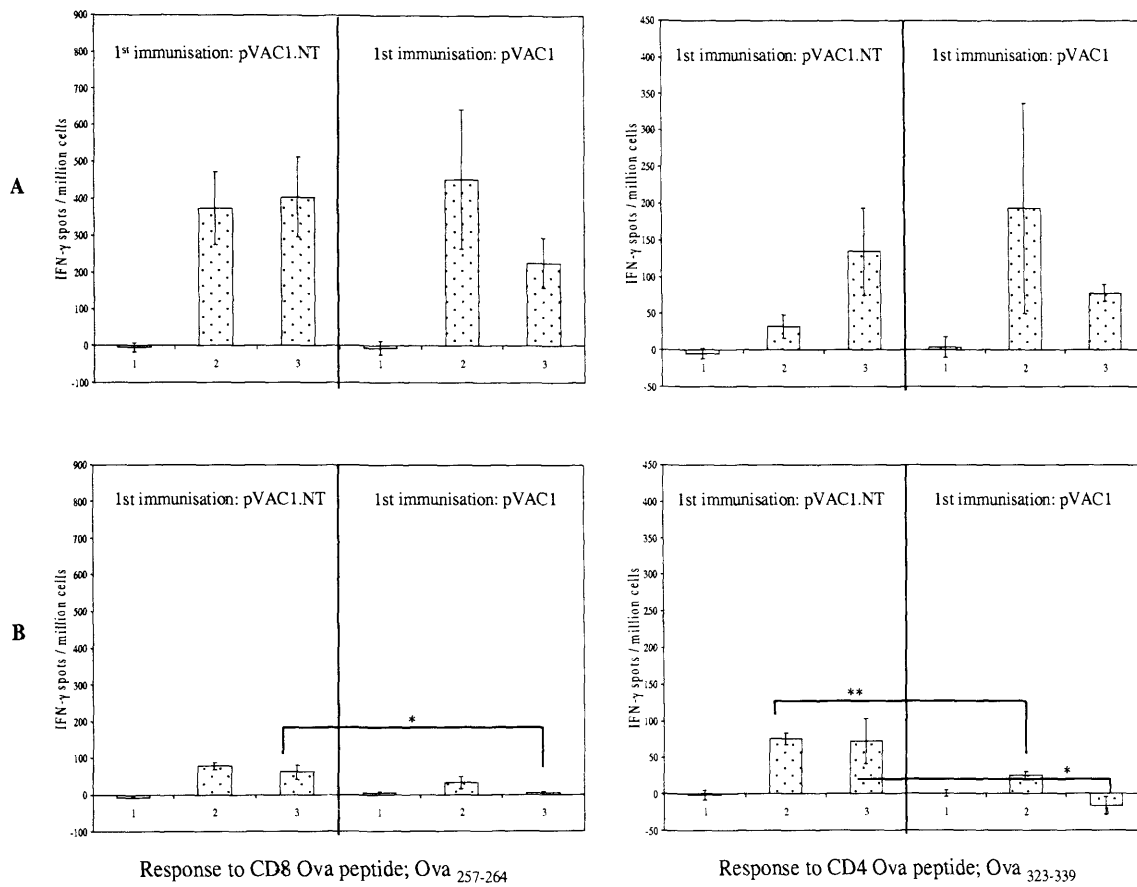


Figure 7.2. OVA specific IFN- γ producing CD8+ and CD4+ T cells detected by ELISPOT. Mice were primed with pVAC1.NT or pVAC1 and boosted on day 28 with cartridges containing NT/empty (group 1), NT/OVAc (group 2) or NT/OVAs (group 3). Spleens were collected for analysis at day 7 PB. Initial and repeat experiments are shown in panels A and B, respectively. For ELISPOT analysis, results are reported as mean of individual means \pm SEM of 3 mice/group. Stars on top of groups represent Student's T-test comparison with corresponding groups (* $p < 0.05$, ** $p < 0.01$).

7.3.1.1. Effect of a pre-existing NT response on NT and OVA responses

Mice were immunised with pVAC1.NT or pVAC1 and boosted 28 days later with groups 1, 2 or 3 (Figure 7.1). In the initial experiment (row A), results show a much higher level of NT specific IFN- γ secretion associated with groups which were primed with pVAC1.NT compared with groups primed with pVAC1. In the pVAC1 and the pVAC1.NT primed mice, similar CD8+ NT responses were obtained in all three groups. In the repeat experiment, greater CD8+ NT responses were also obtained in the pVAC1.NT primed mice (Figure 7.1, row B). However, unlike the initial experiment, the group immunised with NT/OVA (i.e. group 2) yielded a significantly greater level of IFN- γ secretion than the group immunised with pVAC1.NT only (i.e. group 1). The IFN- γ response to the NT CD4 peptide in the repeat experiment for the pVAC1.NT primed groups showed a similar trend to the CD8 peptide analysis. Group 1 yielded less IFN- γ than group 2 while groups 2 and 3 recorded similar levels of IFN- γ production (i.e. ~85, ~190 and ~225 IFN- γ spots/million cells for groups 1, 2 and 3, respectively). The main conclusions from the data are that a single dose of OVA does not result in OVA having a suppressive effect on the NT response.

The CD8+ and CD4+ OVA specific responses are shown in Figure 7.2. As observed with the NT CD4+ and CD8+ responses, the repeat experiment (row B) yielded much lower levels of IFN- γ secretion compared with the initial experiment. In both experiments, the CD8+ and CD4+ responses showed a similar trend. Additionally, the CD4+ responses were generally much smaller and more variable than the corresponding CD8+ responses. In the repeat experiment, there were significant differences in CD8+ and CD4+ response between the NT primed NT/OVAs boosted group and the pVAC1 primed NT/OVAs group. A similar result was obtained when the corresponding NT/OVA groups were compared for the NT CD4+ response. These results suggest that the OVA response is not affected or suppressed by a pre-existing NT response, but rather that the NT response may help a weak OVAs response.

7.3.1.2. Effect of a pre-existing OVA response on NT and OVA responses

Mice were primed with OVAs, OVAc or empty plasmid and boosted 28 days later with groups 1, 2 or 3. NT and OVA CD8+ and CD4+ IFN- γ specific secretion was assessed at day 7 PB. CD8+ specific responses to NT were measured in the initial and repeat experiments while CD4+ specific responses were measured in the repeat experiment only (Figure 7.3). For both experiments, the presence of OVA memory (i.e. pVAC1.OVAc and pVAC1.OVAs primed groups) resulted in a significantly lower CD8+ specific IFN- γ response in groups boosted with group 2 and group 3 compared with groups boosted with group 1. This is particularly well illustrated in the OVAc primed group 2 boosted groups in both experiments where the NT response was completely abrogated. As in Figure 7.1, the IFN- γ CD4+ NT response measured in the repeat experiment was low and variable. Overall, the data demonstrates that priming and boosting with OVA (2 immunisations) results in a suppression of the NT response. Furthermore, pVAC1.OVAc is more suppressive than pVAC1.OVAs.

The corresponding CD8+ and CD4+ OVA responses are shown in Figure 7.4. Results revealed that for both CD8+ and CD4+ responses, the pVAC1.OVAc primed, group 2 boosted group yielded a much greater level of IFN- γ secretion than the pVAC1.OVAs primed, group 3 boosted group in both experiments. This data suggests that pVAC1.OVAc is a more potent immunising plasmid than pVAC1.OVAs. This experiment showed an usually high level of “background” OVA response in mice primed with pVAC1 and boosted with pVAC1.NT which was not explained.

7.3.1.3. Effect of OVA dose response on NT and OVA responses

Mice were primed and boosted with cartridges containing variable amounts of OVAc or OVAs plasmid combined with a constant amount of NT plasmid. NT CD8+ and OVA CD8+ and CD4+ IFN- γ specific secretion was monitored at day 7 PP and PB. For the NT

response, results revealed that, in general, as the level of OVA in the immunising groups

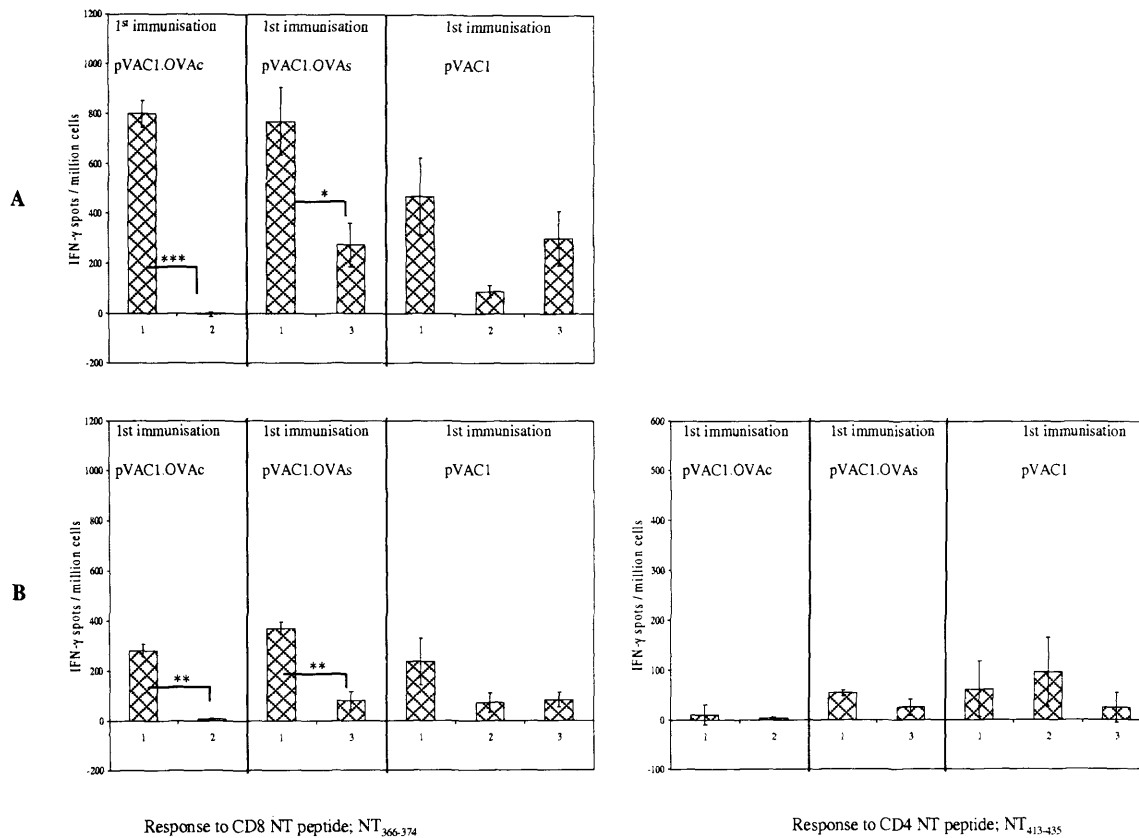


Figure 7.3. Assessing the effect of a pre-existing OVA response on the CD8+ and CD4+ NT response by IFN- γ ELISPOT. Mice were primed with OVAc, OVAs or pVAC1 and boosted on day 28 with cartridges containing NT/empty (group 1), NT/OVAc (group 2) or NT/OVAs (group 3). Spleens were collected for analysis at day 7 PB. For ELISPOT analysis, results are reported as mean of individual means \pm SEM of 3 mice/group. Initial and repeat experiments are shown in panels A and B, respectively. Stars represent Student's T-test comparison with corresponding groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

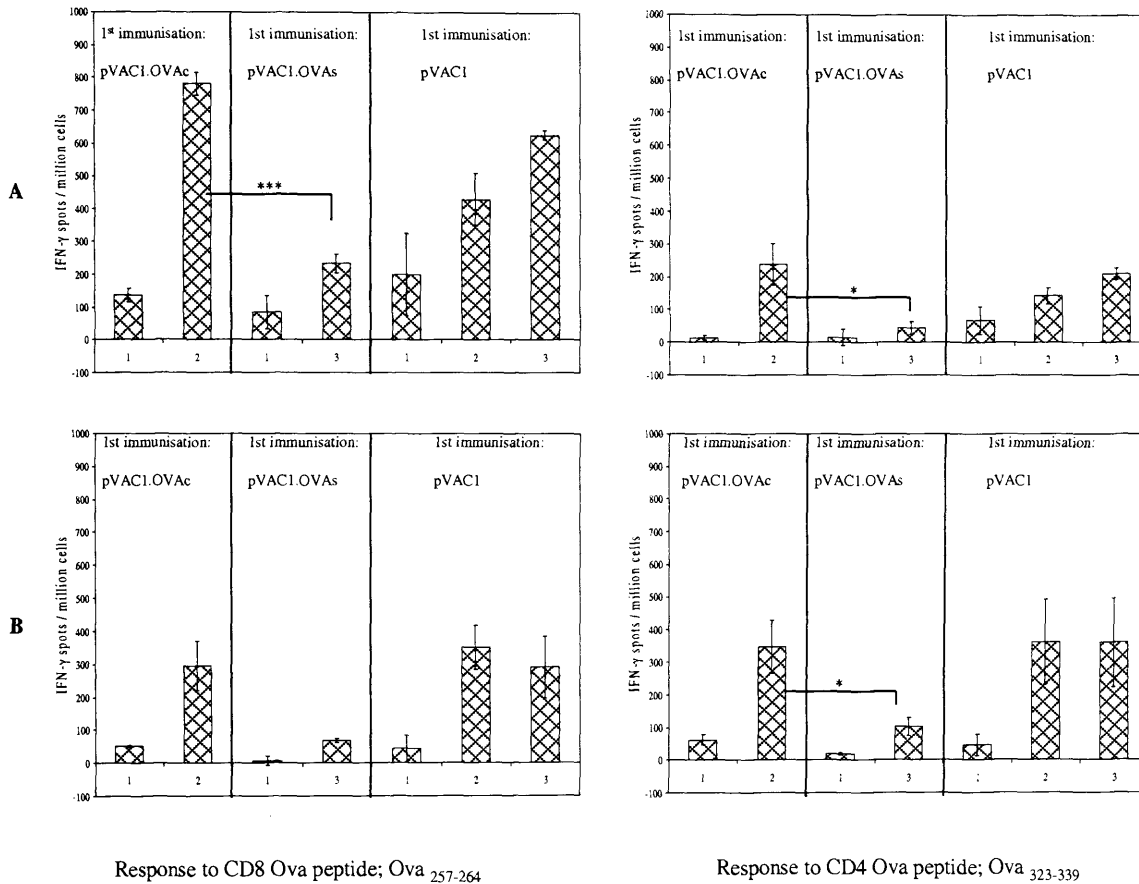


Figure 7.4. Assessing the effect of a pre-existing OVA response on the CD8+ and CD4+ OVA response. Mice were primed with OVAc, OVAs or pVAC1 and boosted on day 28 with cartridges containing NT/empty (group 1), NT/OVAc (group 2) or NT/OVAs (group 3). Spleens were collected for analysis at day 7 PB and assessed for cytokine secretion using IFN- γ ELISPOT. Results are reported as mean of individual means \pm SEM of 3 mice/group. Initial and repeat experiments are shown in panels A and B, respectively. Stars represent Student's T-test comparison with corresponding groups (* $p < 0.05$, *** $p < 0.001$).

decreased, the NT response increased (Figure 7.5). For the CD8+ PP data, this effect was only apparent in the OVAc containing groups (i.e. groups 2–2.2) in the repeat experiment. However, following boost, the magnitude of the NT response increased and the OVA dose response effect was observed in both OVAc and OVAs containing groups. When corresponding OVA groups were compared PB (i.e. groups 2 and 3, groups 2.1 and 3.1 etc.), the level of IFN- γ activity associated with the OVAs groups was generally observed to be greater than that obtained in the OVAc containing groups indicating that pVAC1.OVAc was more inhibitory than pVAC1.OVAs for the CD8+ NT response. Additionally, animals immunised with group 1 gave greater amounts of IFN- γ secretion compared with any of the OVA containing groups. OVA therefore exerted an inhibitory effect on NT even when there is less OVA plasmid than NT plasmid in the immunising groups. Finally, at day 7 PB, the IFN- γ specific CD8+ response obtained in groups 4 and 5 (containing plasmid encoding HIV-Reverse Transcriptase, Nef and Gag protein fusion (HIV-RNG) and human papilloma virus E6 protein (HPV-E6), respectively) was greater than that obtained in the corresponding OVAc group but less than NT alone in both experiments. Like pVAC1.OVAc, plasmids HIV-RNG and HPV-E6 encode cell retained antigen.

The corresponding CD8+ and CD4+ OVA specific responses are shown in Figure 7.6. Results demonstrated that, as the level of OVA plasmid in the immunising groups decreased, so did the OVAs response. The OVAc response did not show this trend but instead remained maximal at all concentrations. The level of CD8+ and CD4+ specific IFN- γ secretion associated with the OVAc groups were generally greater than that associated with the corresponding OVAs groups in both experiments at the day 7 PB time-point.

Together, the NT and OVA analyses indicate that OVAc suppressed the NT response to a greater extent than OVAs. For both OVAs and OVAc, some inhibition of the NT response occurred even when there was less OVA plasmid than NT plasmid in the immunising groups. As the level of OVAs plasmid in the immunising groups decreased, so too did the

resulting OVAs response. This did not happen for OVAc again suggesting that this plasmid is a strong immunogen.

7.3.2. Effect of plasmid linkage on NT and OVA responses

In these experiments, “linked” DNA cartridges refers to the presence of the NT and OVA encoding plasmids on the same gold particles, in the same cartridge and delivered to the same immunisation site (i.e. the standard DNA cartridges used in all PMDD experiments thus far). “Unlinked” DNA cartridges refer to the presence of the NT and OVA encoding plasmids on different gold particles, in different cartridges and delivered to different immunisation sites. The preparation of linked and unlinked DNA cartridges is described in section 2.5.2 and represented in Figure 2.4.

CD8+ specific responses to linked and unlinked DNA cartridges are shown in Figure 7.7. Mice were primed and boosted with groups 1, 2 or 3 (linked or unlinked) along with groups containing pVAC1.OVAc only or pVAC1.OVAs only and NT specific IFN- γ secretion was measured at day 7 PP and PB. Results revealed that at day 7 PP, groups primed with linked and unlinked plasmid gave similar levels of IFN- γ . However, after boost, the groups immunised with the linked DNA (groups 2 and 3) yielded less IFN- γ secretion than the corresponding unlinked DNA groups. Unlinked OVA did not induce suppression of NT in any group. As before, OVAc was a stronger suppressor of NT than OVAs. Finally, animals immunised with the linked group 1 gave significantly greater amounts of IFN- γ secretion compared with the linked groups 2 or 3 in both experiments.

The corresponding CD8+ and CD4+ OVA specific responses are shown in Figure 7.8. Generally, PB results from the linked data revealed that the OVAc containing groups elicited stronger IFN- γ responses than the OVAs containing groups. The OVAc only group yielded a significantly greater level of CD8+ and CD4+ specific IFN- γ secretion compared with the OVAs only group in the repeat experiment. For the CD8+ responses, the group 2 and the group 3 linked groups showed similar IFN- γ activity to the corresponding OVA

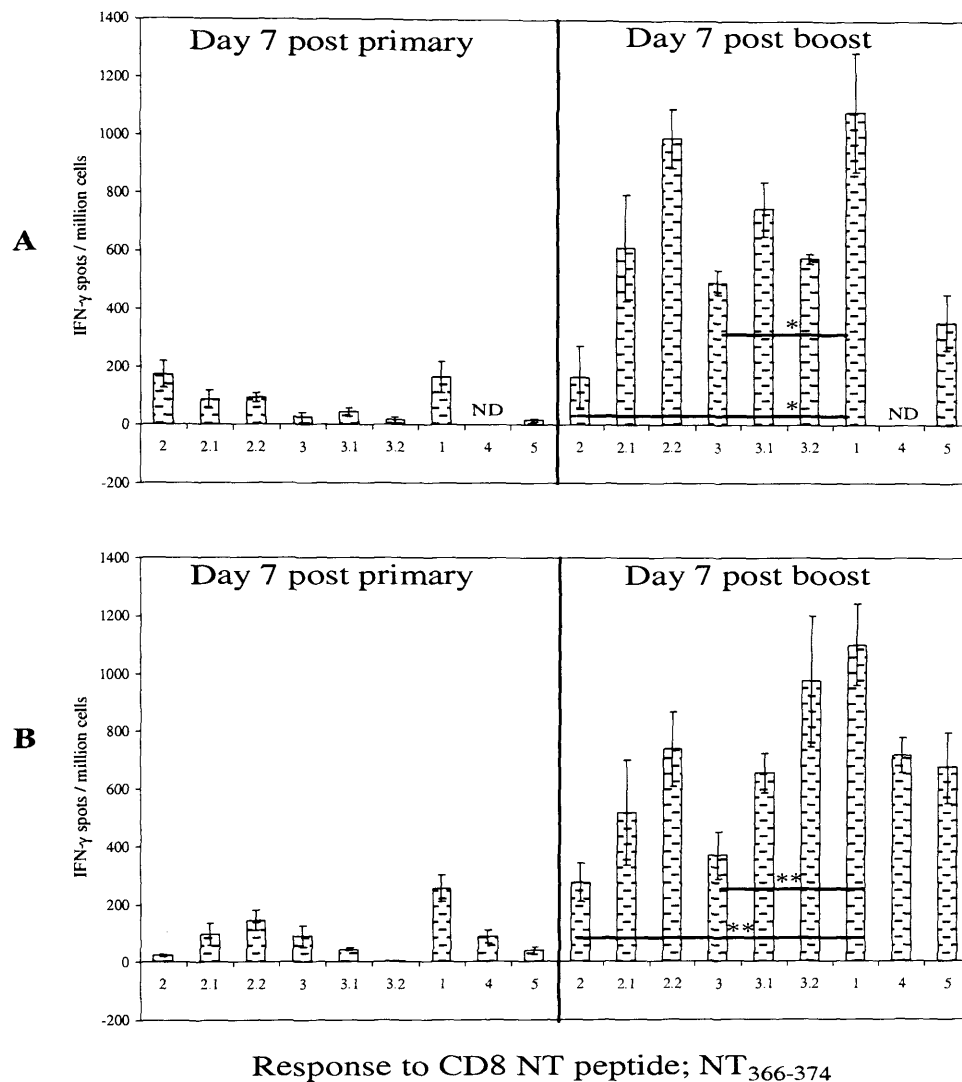


Figure 7.5. Effect of OVA dose response on the CD8+ NT response. Mice were primed and boosted (day 28) with cartridges containing variable amounts of OVAc or OVAs combined with a constant amount of NT. Spleens were collected for analysis at day 7 PP and PB and assessed for cytokine secretion using IFN- γ ELISPOT. For ELISPOT analysis, results are reported as mean of individual means \pm SEM of 3 mice/group. Initial and repeat experiments are shown in panels A and B, respectively. Stars represent Student's T-test comparison with corresponding groups (* $p < 0.05$, ** $p < 0.01$). ND: not done.

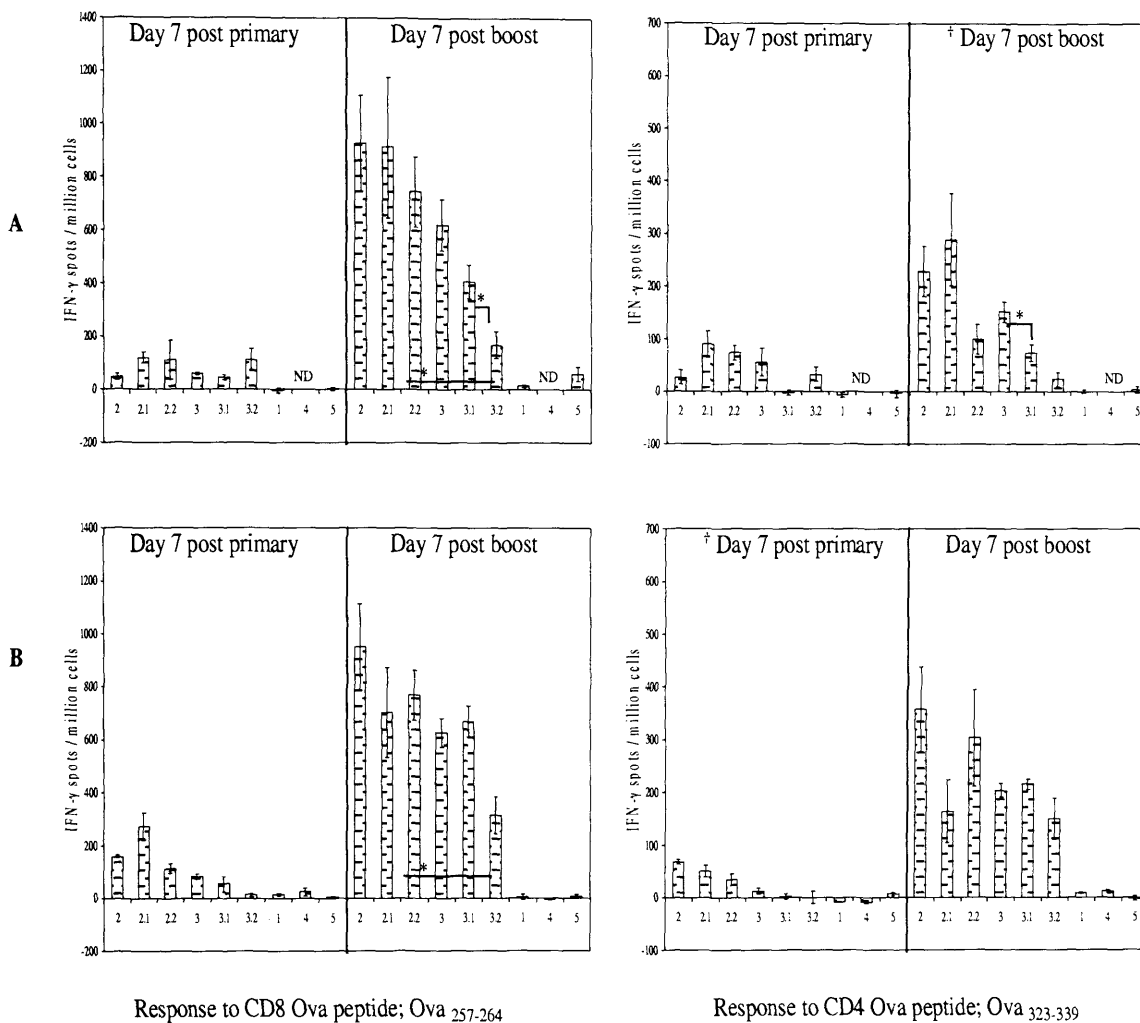


Figure 7.6. Effect of OVA dose response on the CD8+ and CD4+ OVA response. Mice were primed and boosted (day 28) with cartridges containing variable amounts of OVAc or OVAs combined with a constant amount of NT. Spleens were collected for analysis at day 7 PP and PB and assessed for cytokine secretion using IFN- γ ELISPOT. For ELISPOT analysis, results are reported as mean of individual means \pm SEM of 3 mice/group. Initial and repeat experiments are shown in panels A and B, respectively. Stars represent Student's T-test comparison with corresponding groups (* $p < 0.05$). † ELISPOT performed on splenocytes which were frozen on day of spleen harvest and thawed at a later date for analysis. ND: not done.

only groups in the initial experiment and in the case of group 2, greater activity than the OVAc only group in the repeat experiment after boost. This suggests that the presence of NT may positively influence the OVA response. Finally, for the CD8+ response, the OVAc linked group showed a significantly higher level of IFN- γ secretion compared with the corresponding unlinked group in the repeat experiment. In summary, the data indicates that OVA suppression of the NT response requires plasmid linkage. In contrast, the OVA response itself is not negatively affected by plasmid linkage. Indeed, delivering NT and OVA plasmids in a linked manner may benefit the OVAc response.

7.4. Discussion

The interaction between the NT and OVA immune responses was examined by measuring the CD8+ and CD4+ responses to both antigens. For NT, the response to the CD8 and CD4 immunodominant epitopes (D^b restricted amino acid residues 366-374 and $I-A^b$ restricted amino acid residues 413-435, respectively) were measured. For OVA, responses to the CD8 immunodominant epitope (K^b restricted amino acid residues 257-264) and the CD4 immunodominant epitope ($I-A^d$ restricted amino acid residues 323-339) were measured. A number of different immunisation strategies were used which included priming with either pVAC1.NT or pVAC1.OVA prior to co-immunisation, performing prime boost experiments with cartridges containing pVAC1.NT combined with variable amounts of pVAC1.OVA and studying the effect of antigen linkage on NT and OVA responses. Results from these studies suggest that depending on the experimental conditions, both sets of immune responses can compete and co-operate with each other. However, the dominant interaction observed is suppression of the NT response by the OVA response.

7.4.1. Interclonal T-cell competition

7.4.1.1. Competition under conditions of pre-existing immunity

From the experiments performed, there are many lines of evidence indicating that the OVA

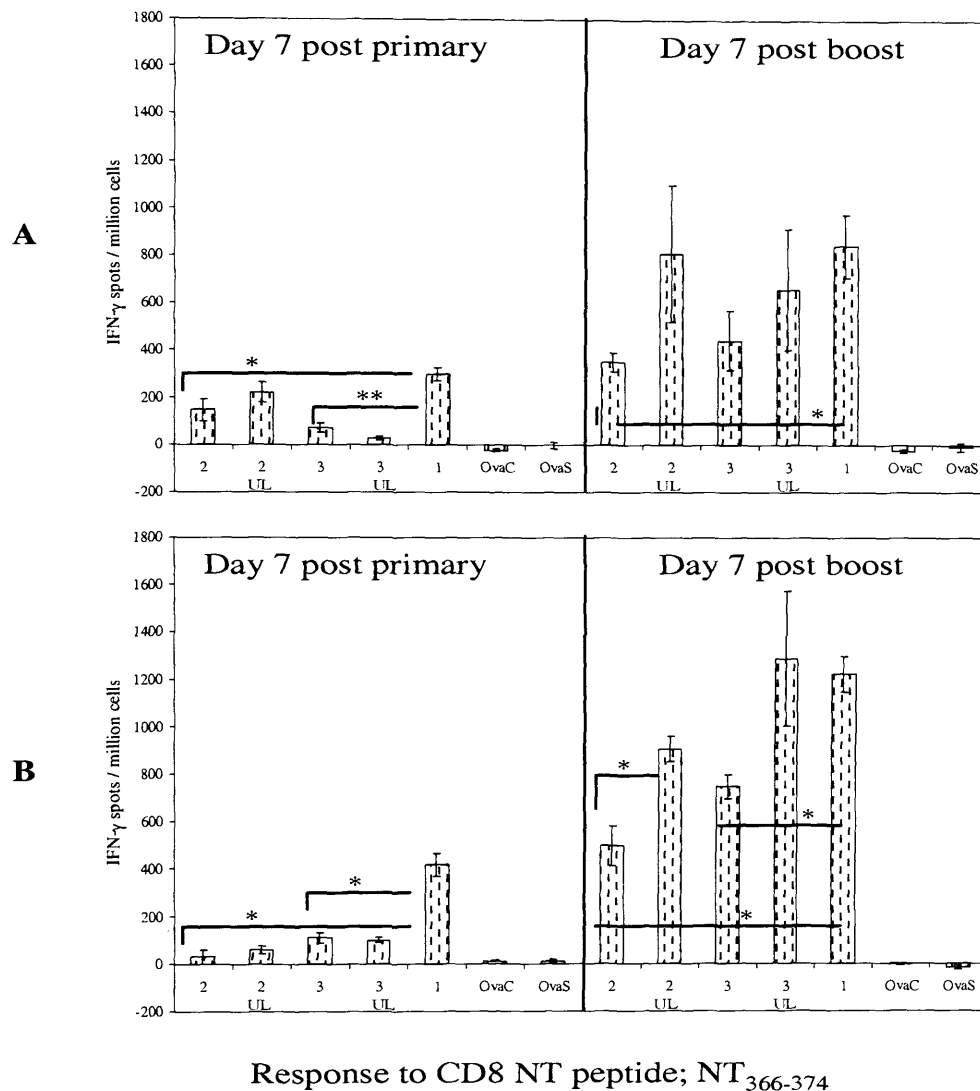


Figure 7.7. Effect of antigen linkage on the NT CD8+ response. Mice were primed and boosted (on day 28) with cartridges containing linked or unlinked NT/empty (group 1), NT/OVAc (group 2) or NT/OVAs plasmid (group 3) as well as groups containing OVAs and OVAc plasmid only. Spleens were collected for analysis at day 7 PP and PB and ELISPOT was used to determine IFN- γ specific activity. Results are reported as mean of individual means \pm SEM of 3 mice/group. Initial and repeat experiments are shown in panels A and B, respectively. Stars represent Student's T-test comparison with corresponding groups (* $p < 0.05$, ** $p < 0.01$).

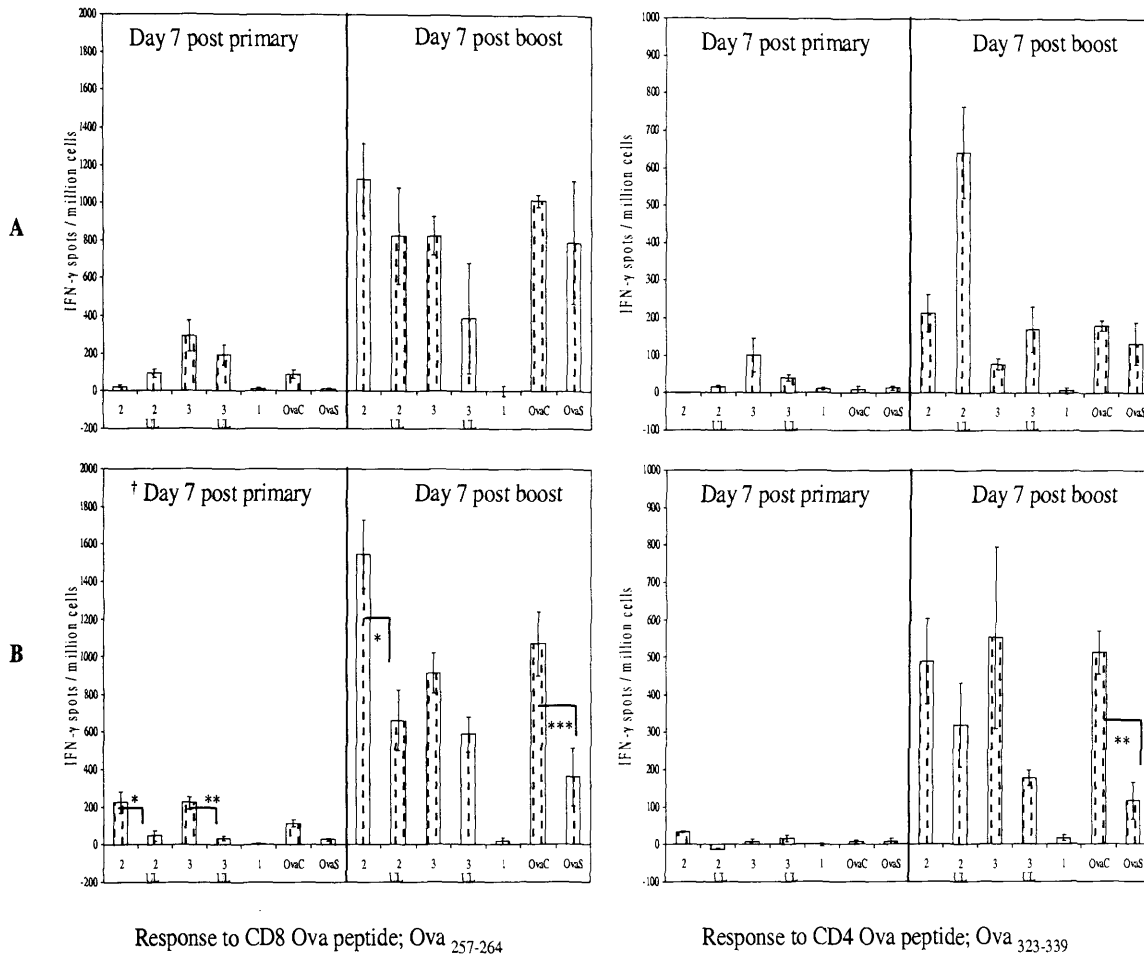


Figure 7.8. Effect of antigen linkage on the OVA CD8+ and CD4+ response. Mice were primed and boosted (on day 28) with cartridges containing linked or unlinked NT/empty (group 1), NT/OVAc (group 2) or NT/OVAs plasmid (group 3) as well as groups containing OVAs and OVAc plasmid only. Spleens were collected for analysis at day 7 PP and PB and assessed for cytokine secretion using IFN- γ ELISPOT. Results are reported as mean of individual means \pm SEM of 3 mice/group. Initial and repeat experiments are shown in panels A and B, respectively. Stars represent Student's T-test comparison with corresponding groups (* $p < 0.05$, ** $p < 0.01$). † ELISPOT performed on splenocytes which were frozen on day of spleen harvest and thawed at a later date for analysis.

and the NT immune responses compete. Firstly, OVA suppression of the NT response was examined in mice which had a pre-existing NT or OVA response established. Others have demonstrated that a pre-existing CTL response to one of the epitopes encoded by a polyepitope DNA construct suppressed the expansion of the CTL responses to other encoded epitopes, restricted by different MHCs (Palmowski *et al.*, 2002). In contrast, there is evidence which demonstrates that a pre-existing CTL response when present in large numbers enhances the priming of other CTL populations restricted by different MHC genes (Sherritt *et al.*, 2000). Here, OVA inhibition of the NT response occurred in mice which had a pre-existing OVA response (Figure 7.3). This was especially evident in the pVAC1.OVAc primed NT/OVAc boosted groups in both experiments where the CD8+ NT response was completely abrogated. In contrast, when NT was delivered with empty vector, a strong NT response was raised. This result is consistent with the findings of many groups (Ge *et al.*, 2004; Rodriguez *et al.*, 2002; Chen *et al.*, 2000c).

Antigenic competition was also observed in the OVAs primed NT/OVAs boosted groups but to a lesser extent than that obtained in the OVAc groups. This difference may be because pVAC1.OVAc is a much more potent plasmid than pVAC1.OVAs and is capable of producing more OVA antigen for presentation. This view is supported by the greater CD8+ and CD4+ OVA responses obtained in the OVAc primed NT/OVAc boosted group compared with the OVAs primed NT/OVAs boosted group (Figure 7.4.) Alternatively, this result may reflect the fact that the OVAs and NT proteins are predominately presented on different DC.

Competition between the OVA and NT responses could occur at the level of the DC (processing/presentation) or at the level of T cell response. As both pVAC1.NT and pVAC1.OVAc encode predominately cell retained antigen and considering the idea that both plasmids end up in the same DC through direct transfection, it is possible that competition for class I processing occurs within the DC (Dick *et al.*, 1994; Eggers *et al.*, 1995). However, it is noteworthy that OVA suppression of the NT response was much stronger after two OVA immunisations. In the case of OVAc, this led to total abrogation of the NT response. In mice with a pre-existing NT response, a single dose of either OVAs or

OVA_c (in the form of NT/OVA boost) had no suppressive effect on the NT response (Figure 7.1). This together with the results from the *in vitro* data presented in the previous chapter argues against processing and presentation as the major source of NT/OVA antigenic competition.

Therefore, it is more likely that competition between the NT and OVA CD8+ T cells occurs at the T cell level. Previous attempts to measure whether interclonal T cell competition occurs *in vivo* have yielded variable results. Some investigators have been successful in demonstrating this type of T cell competition (Kedl *et al.*, 2002) while others have not (Probst *et al.*, 2002; Smith *et al.*, 2000). In the experimental scenario described in this chapter, the OVA memory CTL are present in the DC/T cell microenvironment at a much higher frequency than naïve NT CTL. These cells may act to physically prevent the naïve NT CTL from accessing contact sites on the DC. Alternatively, the OVA memory CTL may act directly to inhibit the naïve NT CTL response. Possible inhibitory mechanisms include T cell suppression mediated by IFN- γ (Rodriguez *et al.*, 2002), down modulation of specific antigen from the DC surface (Hwang *et al.*, 2000; Huang *et al.*, 1999) or antigen extraction from the DC surface (Kedl *et al.*, 2002).

There is also the possibility that the memory OVA response and naïve NT response compete for non-specific factors surrounding the DC/T cell cluster. These non-specific factors may include access to important activation and proliferation related cytokines such as IL-2 (Wodarz and Jansen, 2001), IL-7, IL-15 (Ge *et al.*, 2004) and IL-12 (Eberl *et al.*, 1996) or to numbers of available DC. For the latter, there is also the likelihood that the number of DC available for stimulation in the secondary response is insufficient to sustain activation of the entire OVA memory CTL plus the naïve NT CTL (Lanzavecchia *et al.*, 2002). Limiting numbers of available DC would favour the more abundant OVA response. This is possible considering the immunisation regimen used in these experiments. To this end, it would be interesting to introduce increasing numbers of NT pulsed DC into animals where the OVA T cell response was in vast excess and assess if this led to a concomitant improvement in the NT response.

7.4.1.2. Prime-boost strategies favours OVA competition

The different immunisation strategies used also allowed the interaction between naïve NT and OVA T cell responses and the interaction between memory NT and OVA responses to be examined. The results of these studies (Figures 7.1-7.6) are in agreement with the NT/OVAs *in vivo* dose response data described in the previous chapter (i.e. the presence of OVA results in a reduced NT response compared with immunisation with pVAC1.NT only). There are three prominent features of the interaction between the NT and OVA responses under these conditions. Firstly, the NT response was greatest when pVAC1.NT was delivered with empty plasmid compared with co-immunisation suggesting competition between the NT and OVA responses. Secondly, in the OVA dose response studies, both forms of OVA suppressed the NT response. There was also the indication that suppression occurred when less OVA than NT plasmid was used for immunisation (Figure 7.5) though this experiment needs to be repeated for confirmation of this. Furthermore, the NT response observed in NT/OVAc groups was less than that observed in the NT/OVAs groups, especially after boost. This provides further evidence that OVAc is more inhibitory than OVAs for the NT response. This difference may reflect the relative potencies of both plasmids at generating antigen. The OVAc plasmid is also more potent than the HPV-E6 and HIV-RNG plasmids (all of which encode cell retained antigen) at inhibiting the NT CTL response. Alternatively, it may also indicate that NT/OVA competition is greatest when both antigens are presented on the same APC because OVAs is likely to be secreted and re-presented by neighbouring DC (Kedl *et al.*, 2000; Kedl *et al.*, 2002). Finally, decreasing the amount of OVA plasmid delivered in both cases led to a concomitant increase in the NT response. For OVAs, this data is complimented nicely by the OVA CTL and helper responses which showed a general reduction as the amount of plasmid delivered is decreased (Figure 7.6). The fact that this did not happen with OVAc provides further evidence that competition is greatest when NT and OVA are presented on the same APC.

7.4.1.3. OVA suppression requires linkage

Delivering NT and OVA plasmid in a linked manner resulted in a reduction in the NT response compared with unlinked plasmid delivery (Figure 7.7). This result is in agreement with the previous data and provides further evidence for antigenic competition between the NT and OVA T cell responses following co-immunisation using a prime-boost strategy. The NT response in corresponding linked and unlinked NT/OVAc and NT/OVAs groups were generally similar after the primary immunisation suggesting that little if any intracellular competition occurs between the antigens (Wolpert *et al.*, 1998). However, after boost, unlinking the NT and OVA plasmids resulted in a NT CTL response comparable to the NT/empty linked group. Delivering the plasmids in a linked fashion led to reduced NT responses indicating that OVA suppression requires antigen linkage. Delivering pVAC1.NT to a different immunisation site from pVAC1.OVA means that the NT CTL and helper responses do not have to compete with the OVA response for the non-specific factors involved in T cell activation and proliferation. This is especially important after boost where the OVA memory response is in vast excess and the non-specific factors (IL-2, IL-12, IL-7, IL-15, number of APC and accessibility to MHC and co-stimulatory molecules on the APC) may be limiting. While antigen linkage affects the NT response, it does not appear to reduce the OVA response. Although there is no experimental data on affinities, it may be that the affinity of the OVA TcR for the OVA epitope/MHC complex (SIINFEKL/K^b) exceeds that of the NT TcR for the NT epitope/MHC complex (ASNENMDAM/D^b). If this were the case, the OVA response would dominate the NT response even if both antigens were expressed on the same APC. This may also explain the findings of the OVA dose response studies where OVA suppressed the NT response, even when less OVA than NT plasmid was used for immunisation (section 7.4.1.2).

7.4.2. Interclonal T cell co-operation

Although the NT/OVA interactions are predominately competitive, there is also evidence for T cell co-operation. When a pre-existing NT response was established in mice (Figures 7.1 and 7.2), a greater OVA response was obtained in the NT primed, NT/OVAs boosted

group compared with the pVAC1 primed, NT/OVAs boosted group. This difference reached significance in the repeat experiment. This indicates that the OVA response is not inhibited by a pre-existing NT response, but that this response may help the OVAs response. The mechanism which is responsible for this is unclear but probably involves the activation of, or enhancement of the activation of the OVA presenting DC by the pre-existing NT Th response via the CD40-CD40L pathway (Foy *et al.*, 1996). The memory NT Th cells may stimulate the DC directly leading to the activation of the OVA CTL response. This may be a likely mode of OVA T cell activation especially if the NT and OVA antigens are presented on the same DC following PMDD. Additionally, the NT Th response may provide help to the naïve OVA Th cells allowing those cells to activate the OVA CTL response (Gerloni *et al.*, 2000). Alternatively, the memory NT specific CTL may themselves contribute to DC activation causing the OVA CTL response to be launched (Mintern *et al.*, 2002). If the NT CTL precursor frequency is high, then these cells can induce general activation of DCs, even those which did not present the NT antigen (Ruedl *et al.*, 1999). In this event, there would be no requirement for the NT and OVA antigens to be expressed on the same DC. The second line of evidence supporting T cell co-operation is the greater OVA response obtained in the linked NT/OVAc group compared with the corresponding unlinked NT/OVAc group after boost (Figure 7.8). It appears as if under these immunisation conditions (9:1 ratio of pVAC1.OVA to pVAC1.NT), the OVA response benefits most when NT antigen is available in the same DC/T cell cluster as OVA antigen. The mechanisms responsible for the NT/OVA co-operation may be those previously discussed.

7.5. Conclusions

In this chapter, the role of antigenic competition and co-operation in shaping the NT and OVA immune responses following co-immunisation was investigated. Results suggested that, under these conditions, the interaction between the NT and OVA immune responses was predominately a competitive one. The main findings were that

- The OVA response suppresses the NT response but this inhibition requires at least two doses of OVA (e.g. a prime-boost immunisation strategy).
- OVA suppression may occur even when animals were immunised with less OVA than NT plasmid.
- Suppression requires antigen linkage.
- Suppression is not reciprocated by the NT response.

Although much less prominent, there was also evidence that NT/OVA T cell co-operation occurred and that this also required antigen linkage.

The experimentally contrived conditions generated here may not mimic an *in vivo* situation. However, this data does provide evidence that T cell responses directed against antigens of different specificity can simultaneously interact in a positive and negative manner. The outcome is a reflection of the balance between these opposing forces. It also highlights the implications when two antigens are presented on the same or different DC. For vaccinologists interested in PMDD technology, it demonstrates ways of steering the immune response to a desired outcome. These include simple procedures like manipulating the design of the delivery strategy to more complicated interventions like altering the plasmid construct to produce secreted or cell retained antigen. For a technology like PMDD which is currently struggling to reach the levels of efficacy required for prophylaxis and especially therapy, it is important to optimise these parameters.

Chapter 8

General discussion

8.1. Introduction

The research performed in this thesis addresses issues surrounding PMDD on two different levels. Firstly, the development of an *in vitro* system incorporating PMDD as the means of plasmid delivery along with performing parallel *in vivo/in vitro* studies provided a means to dissect gene gun immunisation into its individual component parts. The comparative similarities and differences observed serve to highlight the technical aspects of the technology which contribute to the overall efficacy of the delivery system *in vivo*. Secondly, issues surrounding dual plasmid immunisation were assessed. The interactions between the immune responses formed by two different antigens following plasmid co-delivery were investigated using different immunisation strategies. Insights into the nature of these immune interactions and the mechanisms of regulation are provided. The impact of this work on the design of future vaccine strategies is discussed.

8.2. Technical aspects of particle mediated-DNA delivery and *in vivo* implications

8.2.1. Requirement for improved transfection efficiency

Many investigators have cited the need for improved DNA vaccine potency in prophylactic or therapeutic models (Reyes-Sandoval and Ertl, 2001). Related to this requirement is the need for an increased level of transfection *in vivo* after PMDD. Evaluation of the parameters associated with PMDD *in vitro* (see chapter 3) suggests that mostly, it is the intrinsic transfectability of the cell itself which dictates transfection efficiency of the target. The effect of many different ballistic variables on transfection efficiency was examined and only the delivery pressure was observed to be of notable importance. This is consistent with the views of many investigators who report optimal delivery pressures varying from 100-1350 psi, depending on the cell line transfected (Novakovic *et al.*, 1999; Murphy and Messer, 2001). Significantly, there was evidence of an interactive relationship between the different ballistic variables for each cell line examined. These findings are similar to those of other investigators (Klein *et al.*, 1987; Heiser *et al.*, 1994) and serve to reinforce the need

for these conditions to be empirically defined for each device used and each cell line transfected.

The general rules which govern PMDD *in vitro* can be extrapolated to the *in vivo* delivery of DNA. Intra-dermal gene gun delivery results in the transfection of both somatic bystander cells and the resident DCs in the skin, the Langerhans cells. The preponderance of reports suggests that the direct inoculation of the latter is the predominant mechanism which leads to DC antigen loading and immune response induction (Timares *et al.*, 2003; Porgador *et al.* 1998). However, only a small number of DCs are present in the transfected area of skin and consequently, the majority of cells transfected are bystander keratinocytes. This leads to the type 2 profile commonly associated with gene gun vaccination (Feltquate *et al.*, 1997). In order to increase the number of directly transfected DC, many strategies have been employed to improve targeting to or increase expression within DCs as outlined in section 1.4.1.5. Indeed, some of these approaches such as the use of the DC specific fascin gene promoter have resulted in the propagation of type 1 immunity characterised by IFN- γ producing Th cells and the production of IgG2a antibodies (Sudowe *et al.*, 2003). Combining strategies that focus transgene expression and antigen production within DC with PMDD transfection conditions which are specifically optimal for DC would further enhance CD8+ T cell mediated immunity. Although, PMDD transfection of murine BMDC *in vitro* was not successful in these studies, there is much evidence supporting direct inoculation to murine or human DC *in vivo* (Condon *et al.*, 1996; Larregina *et al.*, 2001). Indeed, DC specific promoters in conjunction with reporter genes may be directly employed to elucidate the optimal conditions for PMDD to such cells *in situ*.

8.2.2. Impact of antigen dose

The role of antigen dose in shaping the immune response was investigated by firstly varying the DNA dose administered and secondly by varying the amount of protein transcription in the context of a constant DNA plasmid dose. Although the *in vitro* analysis is limited by lack of sensitivity, both the immunisation and promoter dose response studies presented in chapter 5 (co-delivery of pVAC1.NT and empty plasmid) demonstrated a

broadly positive correlation between level of antigen presented and activity of the responding CD8+ T cell line. This data is consistent with the *in vivo* immunisation dose response curves (under conditions of single and dual immunisation), especially the post primary data and with the findings of other groups who investigated this relationship *in vitro* (Pihlgren *et al.*, 1996) and *in vivo* (Murata *et al.*, 1996; Kundig *et al.*, 1996).

The correlation observed in the *in vitro* promoter dose studies is in agreement with investigators who have addressed the role of antigen dose *in vivo*. By employing a recombinant vaccinia virus system which generated a wide range of OVA SIINFEKL surface epitope expression, Wherry and colleagues assessed the influence of antigen density (during primary immunisation) on CD8+ T cell priming (1999) and on the magnitude of the memory response (2002). In both studies, the level of antigen density was observed to be proportional to the CD8+ T cell response. Interestingly in both cases, this relationship broke down when excessive levels of epitope were presented ($\sim 6 \times 10^4$ epitopes/cell). Likewise, Bullock *et al.* (2000) obtained similar results following immunisation of mice with DCs presenting different densities of epitopes derived from human tyrosinase and glycoprotein 100. Again, the magnitude of the responding CD8+ T cell population was greatest when a less than maximal antigen density was used for activation.

However, there are certain characteristics of the *in vivo* antigen dose response that the *in vitro* assay fails to represent. For example, the immunisation dose response curves described in chapter 5 exhibits a linear type relationship at the PP time-points and at day 7 PB. At day 14 PB, this shifts to a predominately threshold type profile. This change may be explained by epitope density based selection of high affinity T cells in the memory response. Bullock *et al.* (2003) demonstrated that variations in cell surface epitope density led to the generation of CD8+ T cells with different avidities. At lower epitope densities, antigen specific T cells with higher TcR affinities were preferentially selected. Others have also documented a reciprocal relationship between the magnitude of the responding population and TcR avidity with changing antigen levels (Wherry *et al.*, 2003). Although TcR affinity was not measured *in vivo* or *in vitro* in the present study, the threshold type curve obtained at the final PB time-point may be representative of a qualitatively

heterogeneous response where the avidity of the responding population generated at lower antigen levels compensates for lack of magnitude obtained when greater levels of antigen are available for presentation. One prediction of this model would be that higher levels of antigen would allow more low avidity T cells to enter into the response and therefore encourage a higher degree of intraclonal competition. In contrast, T cells with high affinity TcR created as a consequence of low level antigen expression would be much more dominant in their inhibition of other antigenic specific T cells allowing only high avidity T cells to preferentially expand into an effector population.

Alternatively, a much simpler but less likely explanation may be that there is a finite resource of cytokines and growth factors available in the immune micro-environment to support an immune response. Following delivery of a single plasmid, the resulting CD8+ T cell population expands until this supply becomes exhausted. The response obtained at lower antigen levels may represent CD8+ T cells which have undergone more divisions than cells stimulated by a higher antigen dose. While this reasoning fits well with the data of Pertmer *et al.* (1995), it does not explain why the antigen dose-response curves are relatively linear at the PP and day 7 PB time-points. Irrespective of the mechanism responsible, the apparent similarities and differences between the *in vitro* and *in vivo* data suggest that the antigen dose-response relationship under conditions of single plasmid immunisation is not always linear. Importantly for gene gun delivered DNA vaccines, there may be an optimal antigen density that results in the generation of a quantitatively and qualitatively effective immune response.

8.2.3. Does intracellular antigenic competition occur *in vivo*?

There are many factors which may contribute to antigenic competition within the APC *in vivo*. Eggers *et al.* (1995) and Niedermann *et al.* (1996) demonstrated the dependency of epitope generation on the cleavage preference of the proteasome. Levitsky and colleagues (1997) documented a role for mechanisms involved in transport of immunogenic peptides to the cell surface. Additionally, there is also evidence that the peptide affinity for MHC class I (Chen *et al.*, 1994; van der Most *et al.*, 1996) and stability of these complexes

(Levitsky *et al.*, 1996) influences the immunodominance of certain peptide antigens and subsequently the resulting T cell response.

The *in vitro* results presented in chapter 6 suggested that the presence of the OVA plasmid had no effect on the magnitude or sensitivity of the NT response, irrespective of whether the OVA was retained in the cell in which it was synthesised or whether it was secreted. Consistent with this was the finding that the lowest dose of plasmid capable of eliciting an NT specific response in single and dual immunisations *in vivo* was the same (0.5 ng). Additionally, evidence from the *in vivo* co-immunisation studies (see chapter 7) indicated a requirement for more than one immunisation for OVA suppression of NT. Under conditions of pre-existing OVA immunity, a second dose of OVA led to a large reduction or total abrogation of the NT response. When a pre-existing NT response was present, a single dose of OVA had no effect on the NT response. Collectively, these data suggest that in the present system at least, intracellular antigenic competition is not functionally important and the source of antigenic competition happens at the T cell level (see section 8.3). This hypothesis is in agreement with Wolpert *et al.* (1998) and Grufman *et al.* (1999b), both of whom attribute interactions between the responding T cell populations and not APC associated mechanisms as the cause of interclonal competition. From a vaccine perspective, this is an important observation and lends support to the approach of delivering multiple plasmid vectors (especially co-delivery of plasmids encoding molecular adjuvants such as IL-2, IL-12 or GM-CSF with the plasmid encoding the antigen of interest) as a feasible vaccination strategy.

8.2.4. Professional and non professional cells present epitopes differently

The disparities observed *in vitro* and *in vivo* throughout this work may in part be due to the differential processing and presentation of the NT immunodominant epitope (D^b restricted ASNENMDAM peptide) by a fibroblast cell line *in vitro* and DCs *in vivo*. Of special relevance to this work is the research by Butz and Bevan (1998b) who investigated the presentation of D^b LCMV peptides by MC57 cells and a DC line (JawsII). They found that when virus infected MC57 cells were used to re-stimulate spleen cells, the responding CTL

lines failed to react to the two D^b restricted immunodominant epitopes but was lytic for targets expressing the normally subdominant epitope. In contrast, LCMV infected JawsII cells stimulated CTL lines reactive against the immunodominant but not subdominant epitopes. Together, the data suggested that different cell types process protein in quantitatively different ways. This difference was ascribed to either a deficiency in proteolytic activities within MC57s resulting in a much reduced level of immunodominant epitope generation and/or the presence of mechanisms favouring the processing of the subdominant epitope. Heterogeneity of antigen processing and presentation to CD4⁺ T cells by different presenting cell lines was also reported in the hen egg-white lysozyme model (Gapin *et al.*, 1998). Moreover, others have demonstrated that the same antigen can be processed differently by two genetically identical antigen-presenting cell lines (Michalek *et al.*, 1989).

The possibility that there are differences in NT protein processing between MC57s *in vitro* and professional APC *in vivo* would partially explain the lack of sensitivity of the RAG1.F5 splenocytes *in vitro*. Arguing against this hypothesis however is the similar APC:cytokine spot ratios obtained when PMDD transfected and peptide pulsed MC57s were compared (see chapter 4) which suggest that this cell line is relatively proficient at peptide processing and presentation. Irrespective of whether or not there is a problem with presentation of immunodominant epitopes within MC57s, it does highlight an important mechanism *in vivo* which delivery of DNA vaccines by gene gun can exploit. While DC play an important role in focusing the immune response towards a few select epitopes, peptide presentation by a variety of APC types ensures priming of CTL against subdominant epitopes. This leads to a more diverse T cell repertoire important in the elimination of chronic infection (van der Most *et al.*, 1996). Gene gun delivery of plasmid which results in multiple cell types transfected provides an opportunity to avail of this immune mechanism.

8.2.5. Limitations of the *in vitro* model

The main limitation of the *in vitro* model was lack of sensitivity. This was especially evident from the dose response studies where the sensitivity was ~100 fold less than that obtained *in vivo*. This large difference may be attributed to a number of factors associated with either the presenting or responding cells used. One explanation may be the differential processing capabilities of MC57 cells and APC *in vivo* as noted previously. Alternatively, fibroblasts as non professional presenting cells possess no co-stimulatory molecules which are not only important for T cell priming but also play a role in the maintenance of a highly effective memory response (Ludewig *et al.*, 1999). The requirement for co-stimulatory molecules may be critical in this *in vitro* model considering the absence of CD4+ T cell help as well as the usual compliment of cytokines and growth factors normally available *in vivo*.

The relative lack of sensitivity *in vitro* may be a consequence of the culturing process used to generate the responder CD8+ T cell population. There is the possibility that the concentration of peptide used for stimulation and the time interval of stimulation during the culturing process was too high and sub-optimal, respectively (500 nM peptide for 7 days). Alexander-Miller and colleagues (1996) demonstrated that, *in vitro*, culturing cells in high concentrations of peptide resulted in low avidity CTLs with much reduced lytic capacity *in vivo*. Moreover, the same author showed that such populations were more susceptible to AICD by TNF- α receptor II mediated apoptosis (Alexander-Miller *et al.*, 1998). Defining the peptide concentration that is too high or that is optimal for high avidity CTL generation is an empirical exercise specific to each CTL line. Presumably, it is dependent on factors such as peptide affinity for MHC I and the diversity of the T cell repertoire from which the CTL is derived. Because, activated T cells from the RAG1.F5 transgenics were used as responders in the *in vitro* studies, the culturing conditions used should not have affected the avidity of these cells. However, these conditions may have contributed to the production of less responsive RAG1.F5 T cells which would help explain the lack of sensitivity of the *in vitro* model. Indeed, it is most likely that the sensitivity issues associated with the *in vitro* model are due to most or all of the shortcomings discussed.

Overall, the *in vitro* model proved to be relatively limited in its ability to predict *in vivo* responses. The data from the single and dual immunisation dose correlation studies indicated that the *in vitro* model was useful in determining the early stage primary response *in vivo*. After boost however, this relationship deteriorated. These results provide an insight into the differing factors which influence the *in vivo* immune response at the different stages. Early on in the immune response, the most important factor which affects the expansion of the naïve NT specific CD8+ population is the level of antigen presented to these cells by APCs. Therefore, it is not surprising to find that the *in vivo* response is most analogous to the *in vitro* model at this stage as the latter is a two cell system responsive mostly to the level of antigen administered.

After boost, the *in vivo* response is influenced by both the quantity of antigen presented and the quality of the recall response generated during priming. It is characterised by the maturation of the memory response which selects for high avidity T cells. Additionally, due to the presence of competitive interactions at the T cell level (especially under conditions of dual plasmid immunisation), the NT response may be operating under limiting conditions. The poor correlations obtained when the *in vitro* and the PB *in vivo* data are compared (following single plasmid immunisation) are a reflection of the shift from the mechanisms that dominate the primary response to those that dictate the memory response *in vivo* and the failure of the *in vitro* model to account for these. In order to generate an *in vitro* assay which is more representative of the primary and secondary phases of the immune response *in vivo*, the minimal requirement would be the use of professional APCs together with CD4+ T cell help and a source of cytokines for T cell activation and proliferation. Issues such as intracellular competition and the effect of T cell avidity on interclonal T cell competition may be investigated more appropriately if T cell specific for the second immunodominant peptide (i.e. SIINFEKL) was also evaluated.

8.3. Interclonal competition is functionally relevant in dual plasmid immunisation

Because the number of T cells in the mammalian immune system at any one time increases due to ongoing production of new cells from the thymus or expansion during infection, there are an array of homeostatic mechanisms which maintain the sizes of the overall naïve and memory T cell repertoires at a constant level. Such control mechanisms exist at many different levels and stages of the T cell lifecycle and help to guard against the onset of autoimmune diseases and the generation of excessive responses to foreign antigens which may result in immune pathology. For example, CD8⁺ memory cell turnover is predominately governed by cytokines such as the positive contribution of IL-15 (Fehniger *et al.*, 2001) and IL-7 (Schluns *et al.*, 2000) and the negative impact of IL-2 (Ku *et al.*, 2000) and IFN- α (Varga *et al.*, 2001). Following encounter with antigen, the expansion of CD8⁺ effector cells are kept in balance by a number of mechanisms. These include T cell deletion (Critchfield *et al.*, 1994), anergy induction following suboptimal activation (Mondino *et al.*, 1996) and effect of inhibitory receptors such as CTLA-4 (Doyle *et al.*, 2001). In addition, the T cell response is further regulated by the competitive actions of the T cells themselves (Troy and Shen, 2003). Proliferating cells may compete with each other by direct inhibition of neighbouring cells (Kedl *et al.*, 2002) or by competition for limiting resources such as growth factors and cytokines (Ge *et al.*, 2004).

In chapters 6 and 7, the nature of the interaction between CD8⁺ T cells of different specificity was investigated following dual plasmid immunisation. Under the immunisation conditions used, this interaction was found to be predominately competitive. The results obtained highlight important aspects of dual or multi-plasmid immunisation which may underpin vaccine efficacy *in vivo*.

8.3.1. Competition occurs at the T cell level

There was no evidence to indicate that the suppression of the NT response by OVA was as a consequence of antigenic competition within the APC. In contrast, there were many lines of evidence which pointed towards the T cell level as the source of competition. These

findings are in agreement with many investigators who have studied both intraclonal and interclonal T cell competition (Sandberg *et al.*, 1998; Grufman *et al.*, 1999a). Indeed, the mechanisms responsible for intraclonal T cell competition have been documented comprehensively. Early observations from host versus graft responses to MHC antigens (Clark *et al.*, 1981) through to more recent studies using both viral (Butz and Bevan, 1998a) and non-viral (Kedl *et al.*, 2002) models implicate the T cell-APC interface as the zone in which competition takes place. Many investigators have demonstrated that the simple expedient of introducing more antigen expressing APC results in the reduction of intraclonal competition effects (Wolpert *et al.*, 1998; Sandberg *et al.*, 1998). Competition of this kind is orchestrated by the T cells with the highest affinity for the MHC-peptide complex. As discussed in chapter 7, these cells may inhibit the response to other antigen specific T cells by a number of mechanisms including APC killing (Grufman *et al.*, 1999a) or down-regulation (Kedl *et al.*, 2002) or internalization of antigen (Huang *et al.*, 1999) from the APC surface. Irrespective of the means by which competition occurs, the end result is that low affinity T cells are effectively starved of the appropriate levels of antigen required for activation. In this respect, the occurrence of T cell affinity maturation may be regarded as a natural by-product of the process of intraclonal T cell competition.

Whether interclonal CD8⁺ T cell competition exists or not is uncertain. Chen and colleagues (2000c) demonstrated this phenomenon in an influenza model. This is further supported by the work of Kedl *et al.* (2002) who observed competition between the CD8⁺ T cell responses to the dominant and subdominant ovalbumin epitopes following transfer of OT1 T cells. Other investigators however, have concluded that this type of competition does not exist (Butz and Bevan, 1998a; Probst *et al.*, 2002). This apparent contradiction suggests that interclonal CD8⁺ T cell competition may occur but only under certain experimental conditions and in specific experimental models. In the studies where interclonal competition has been observed, there is a requirement that both antigens are presented on the same APC. This is in agreement with the results from the experiments in which a pre-existing OVA response was created and in the prime-boost experiments. Both approaches demonstrated greater suppression of the CD8⁺ NT response by OVA CD8⁺ T cells when NT plasmid was co-delivered with plasmid encoding cell retained OVA

compared with secreted OVA. In addition, the antigen linkage experiments also support this requirement. A prominent feature of all of the experiments in chapter 7 is that the NT CD8⁺ response was greatest when NT plasmid was delivered with empty plasmid compared with OVAs or OVAc plasmid (linked or unlinked). Collectively, this suggests that T cells of different specificity may not only compete for the limited space available at the T cell-DC interface but in the local environment surrounding the DC-T cell cluster for soluble factors such as IL-2, IL-7 and IL-15.

Although not examined in this study, the role of regulatory T cells in suppressing the activation and proliferation of the NT CD8⁺ response (and the OVA CD8⁺ response also) must also be considered. Many reports have demonstrated that depletion of this T cell subset results in much increased responses to foreign antigen. Suvas and colleagues (2003) showed that by removing CD4⁺ CD25⁺ T cells prior to HSV infection, the CD8⁺ T cell effector and memory response to the immunodominant peptide was increased by a factor of up to fourfold. The effect of CD4⁺ CD25⁺ T cells on the response to foreign antigen has also been observed in bacterial (Xu *et al.*, 2003; Belkaid *et al.*, 2002) and tumour models (Shimizu *et al.*, 1999). Regulation may be mediated through the secretion of inhibitory cytokines such as IL-10 (Barrat *et al.*, 2002) or TGF- β (Chen *et al.*, 1998). Their constitutive expression of CD25 allows these cells to compete effectively for IL-2 thus limiting the expansion of other antigen specific T cells. Additionally, CD4⁺ CD25⁺ T cells also express CTLA-4 allowing them to compete for co-stimulatory molecule engagement at the DC-T cell interface (Read *et al.*, 2000). The latter mechanism may be a more significant issue in situations where intraclonal T cell competition is a feature of the immune response, while competition for IL-2 around the DC-T cell cluster may have more of a bearing on the competition between T cells of different antigen specificity. Regardless of the mechanisms responsible for interclonal T cell competition, the results from chapter 7 demonstrate that CD8⁺ T cell competition is functionally relevant and can occur even when standard vaccination strategies such as a PMDD prime-boost approach are employed.

8.3.2. Importance of TcR avidity

Data from the OVA dose response and antigen linkage experiments suggest that the OVA response may have a competitive advantage over the NT response due to the greater affinity of the OVA TcR for MHC/peptide than the NT TcR. This hypothesis gains support from a number of sources who have demonstrated the link between TcR affinity and efficacy of the resulting response. Using MHC I tetramers to stain CD8⁺ T cells specific to an immunodominant *L.monocytogenes* peptide, Busch and Palmer (1999) were the first group to show that the affinity of T cells for antigen increases with the number of rounds of expansion *in vivo*. Similar results were obtained by Savage *et al.* (1999) who revealed a narrowing of the T cell repertoire in the secondary response compared with the primary response and linked this to the selective loss of T cells which dissociated most rapidly from the MHC/peptide complex. The dominance of influenza specific CD8⁺ T cells was attributed to their greater affinity for antigen in humans (Lawson *et al.*, 2001). This effect was also observed by Rees *et al.* (1999) with CD4⁺ T cells. Interestingly, in the latter study, the authors demonstrated an inverse relationship between T cell affinity and antigen dose (i.e. the lower the dose, the higher the affinity) in the secondary but not primary phases of the response.

While the relationship between the competitive ability of a T cell and its dependence on affinity for antigen has direct implications for intraclonal T cell competition, it may also impact on interclonal T cell competition. Although Kedl *et al.* (2002) observed interclonal T cell competition between high affinity transferred OT1 T cells and endogenous T cells of a different specificity, they observed this phenomenon to be much less efficient and require almost a log more transferred cells than was needed to demonstrate intraclonal competition. These findings may provide an explanation for the effect observed in the OVA dose response study in chapter 7 where OVA suppression occurred when less OVA than NT encoding plasmid was delivered. If so, it is possible that T cell avidity has functional implications in modulating the quality and quantity of the immune response to different antigens following dual plasmid immunisation.

8.4. Lessons for future vaccination strategies

The results from the human PMDD vaccine trials performed to date (see section 1.4.1.8) portend a technology which is likely to struggle to reach the levels of efficacy required to confer prophylactic or therapeutic protection. The work presented in this thesis highlights aspects of PMDD that may be exploited in order to increase chances of a successful vaccination strategy. Initially, consideration must be given to the type of immune response that is required for protection or clearance of a particular pathogen before designing an appropriate vaccine strategy. Induction of the incorrect type of immunity (type 1 versus type 2) could lead to an exacerbation of pathogenesis which is undesirable. Delivering the appropriate antigen in a manner which modulates the response towards the desired phenotype is important. The inherent ability of PMDD to transfect many different cell types *in vivo* provides the means to launch cellular and humoral immunity simultaneously. If there is a specific requirement for a type 1 response, then targeting the class I pathway by using optimal PMDD conditions for DC transfection as well as DC specific promoters may be used. As previously discussed, defining the optimal balance between antigen dose and T cell avidity is critical for developing vaccine approaches which selectively induce high affinity T cells.

Important consideration must also be given to the role of interclonal T cell competition in the development of vaccine strategies. The research performed here suggests that competition of this nature has functional relevance following dual plasmid immunisation. Consistent with other investigators, the data also suggests a requirement of T cell competition for co-expression of epitopes on the same antigen presenting cell (Rodriguez *et al.*, 2002; Kedl *et al.*, 2002). The natural bias of an immune response for a relatively small number of immunodominant epitopes may be alleviated by the simple expedient of separating the plasmids encoding the different antigens or epitopes onto different gold beads. This would allow for greater dispersal of epitopes amongst available APC and restrict competition to non specific components of the immune response such as access to cytokines and growth factors. This may benefit many different types of DNA vaccine

approaches but presumably is of particular relevance to multivalent or polyepitope vaccines.

An alternative approach would be delivery of the antigens responsible for the dominant and subdominant epitopes in separate immunisation phases. This idea is not only supported by the results in chapter 7 (i.e. experiments in which a pre-existing OVA or NT response was created) but also by the findings of many other groups (Schirmbeck *et al.*, 2002; Santra *et al.*, 2002). Palmowski and colleagues (2002) demonstrated that a pre-existing response to one of the immunodominant epitopes encoded in the polyepitope vaccine dominated the CTL response to the other epitopes in subsequent immunisations. Moreover the same author also showed that a broad CTL response could be achieved by restricting the use of polyvalent constructs to the initial immunisation and using separate constructs for subsequent boosting. Furthermore, pre-immunisation with a subdominant antigen can greatly reduce the response to an otherwise immunodominant dominant antigen (Schirmbeck *et al.*, 2002). These approaches provide evidence that there are tangible ways of effectively counteracting the contribution of interclonal T cell competition, if necessary.

PMDD is an attractive approach for generating antigen specific immunotherapy. Indeed, it may at some point in the future prove to be the most effective method for conferring prophylactic or therapeutic protection in man. Before this happens, a number of important issues such as optimisation of potency, co-induction of systemic and mucosal immunity, plasmid design and appropriate delivery regimens have to be optimised for the pathogen in question. Fortunately, DNA vaccines are safe with no evidence for significant levels of host genome integration. Furthermore, they are relatively inexpensive to manufacture. The rapid progression of PMDD vaccination from its inception just over a decade ago to its current place in human clinical trials provides hope that this technology can play a decisive role in the prevention and treatment of a wide range of human diseases.

Chapter 9

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